Novel Linkers for the Solid-Phase Synthesis of Peptide Aldehydes

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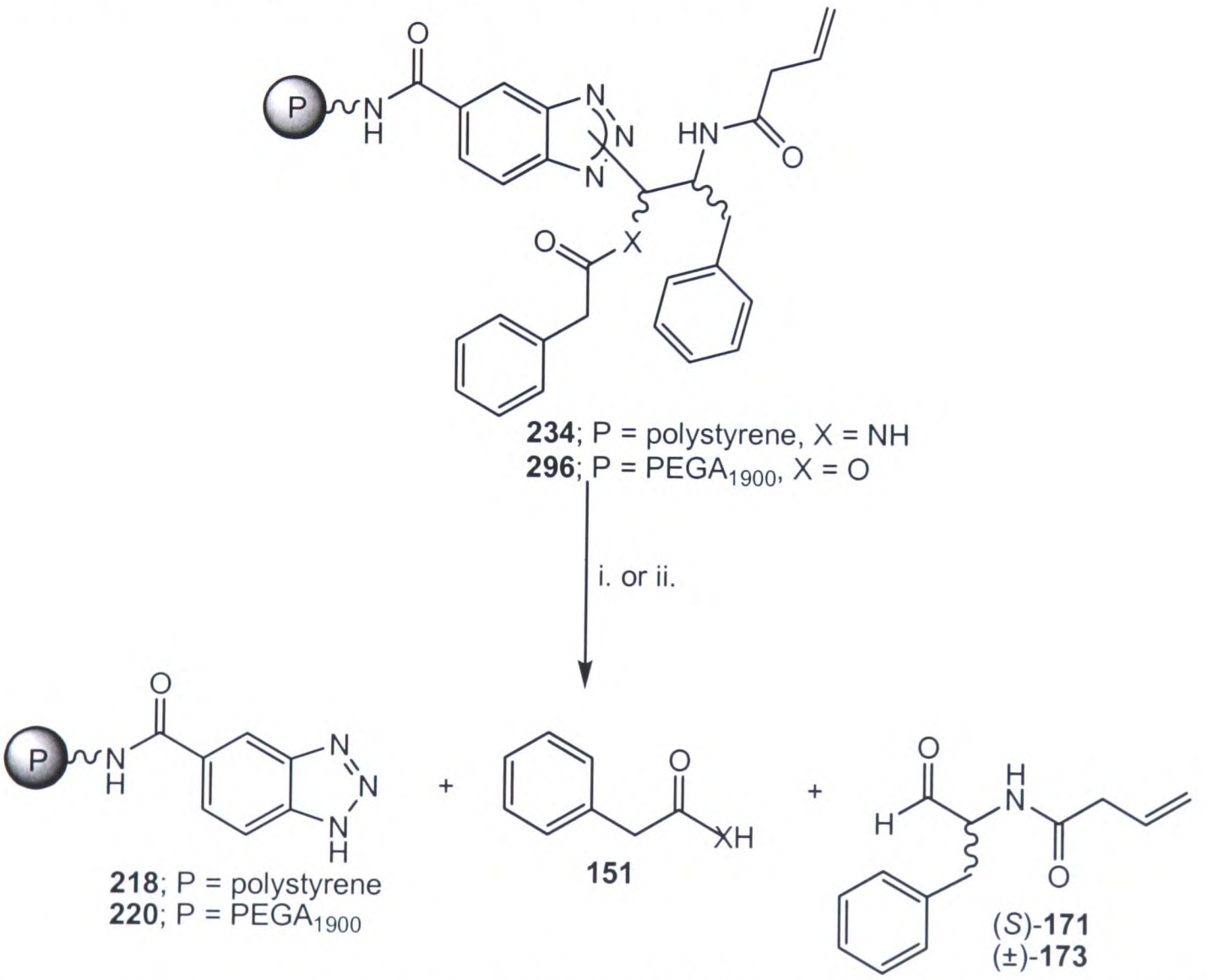
Abstract

Serine and cysteine proteases are potently inhibited by peptide aldehydes. Compared to the plethora of methods available for the solution synthesis of peptide aldehydes, there are relatively few methods for the solid phase synthesis of peptide aldehydes. The development of novel linkers **234/296** for the solid phase synthesis of peptide aldehydes is reported (Scheme 1). When X = NH, **234** the aldehyde **171** can be cleaved with mild acid in >97% enantiomeric excess. When X = O, **296** base hydrolysis results in aldehyde **173** cleavage with complete epimerisation of the α-stereocentre of the aldehyde. The use of *Mucor javanicus* lipase and Penicillin acylase generates the aldehyde **171** in 18 and 31% yield respectively, with complete retention of chiral integrity (>99%). The utility of the linkers **234/296** was demonstrated by the solid phase synthesis of a tetrapeptide aldehyde.



Scheme 1: *Reagents and conditions:* i. TFA/DCM/H_2O (9:10:1) or methanolic ammonia; ii. Penicillin acylase or lipase.

Abbreviations

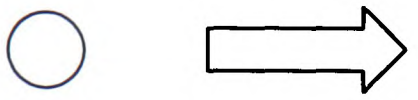
AA	amino acid
Abz	*o*-aminobenzamide
Ac	acetyl
AG-NH_2	ArgoGel^{TM}-NH_2
Ala	alanine
Alloc	allyloxycarbonyl
AM-PS	aminomethyl polystyrene
APS	ammonium persulfate
Boc	butoxycarbonyl
BOP	benzotriazol-1-yloxy-*tris*(dimethylamine)phosphonium hexafluorophosphate
BSA	bovine serum albumin
Bt	benzotriazole
cat.	catalytic
CIAP	calf intestinal alkaline phosphate
cm^{-1}	wavenumber
CM-PS	chloromethyl polystyrene
CPG	controlled pore glass
δ	chemical shift
DCM	dichloromethane
DIC	*N,N'*-diisopropylcarbodiimide
DIPEA	diisopropylethyl amine
DMA	dimethylacetamide
DMAP	dimethylaminopyridine
DME	1,2-dimethoxyethane
DMF	dimethylformamide
DMSO	dimethylsulfoxide
DTT	dithiothreitol
DVB	divinylbenzene
ES-MS	electrospray mass spectrometry
Eq.	equivalents
Et	ethyl
FAB	fast atom bombardment
Fmoc	9-fluorenylmethoxycarbonyl
FRET	fluorescence resonance energy transfer
FT-IR	fourier transform-infrared
g	gram
GalNAc	*N*-acetylgalactosamine
GalT	galactosyltransferase
GlcNAc	*N*-acetylglucosamine
Gly	glycine
h	hours
HATU	*O*-(7-azabenzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate
HBTU	*O*-benzotriazol-1-yl- *N,N,N',N'*-tetramethyluronium hexafluorophosphate

HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HMB-PS	hydroxymethylbenzyl polystyrene
HOBt	*N*-hydroxybenzotriazole
HPLC	high pressure liquid chromatography
Hz	hertz
IBX	2-iodoxybenzoic acid
IR	infrared spectroscopy
J	coupling constant
LC-MS	liquid chromatography mass specrometry
Lit.	literature
M	mol l^{-1}
μ	micro
M^+	molecular ion
MAS	magic angle spinning NMR
MBHA	4-methylbenzhydrylamine
Me	methyl
MeOH	methanol
MHz	Mega hertz
MMP	matrix metalloproteinase
MP	macroporous polystyrene
Mpt	melting point
m/z	mass to charge
NaCl	sodium chloride
NMR	nuclear magnetic resonance
O/N	overnight
PEG	polyethylene glycol
PEGA	polyethylene glycol acyrlamide
Ph	phenyl
Phe	phenylalanine
p.p.m	parts per million
PS	aminomethyl polystyrene
pyBOP	benzotriazol-1-yloxy-*tris*-pyrrolidinophosphonium hexafluorophosphate
RP	reverse phase
R_t	retention time
r.t.	room temperature
s	singlet
sat.	saturated
SPOCC	superpermeable organic combinatorial chemistry
TEMED	*N,N,N',N'*-tetramethylethylene-diamine
TBAI	tetra-*n*-butylammonium iodide
TFA	trifluoroacetic acid
TG	TentaGel
THF	tetrahydrofuran
TLC	thin layer chromatography
TNBS	2,4,6-trinitrobenzenesulfonic acid
TsOH	*p*-toluene sulfonic acid (tosic acid)
UDP	uridine diphosphate
U.V.	ultraviolet

ν	frequency
Val	valine
$Y(NO_2)$	3-nitrotyrosine

Resins

The following symbols have been used as abbreviations for general types of solid supports.



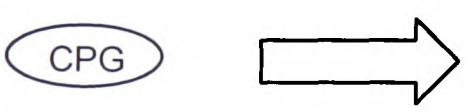
unspecified resin

polystyrene 1% crosslinked with DVB

macroporous polystyrene

Tentagel

Polymer lab $PEGA_{1900}$ resin



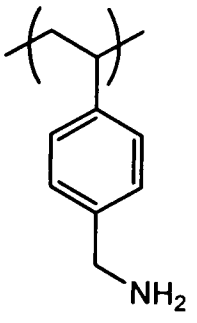
Controlled pore glass

Some of the resins which have been used in the experimental section of this thesis are illustrated below;

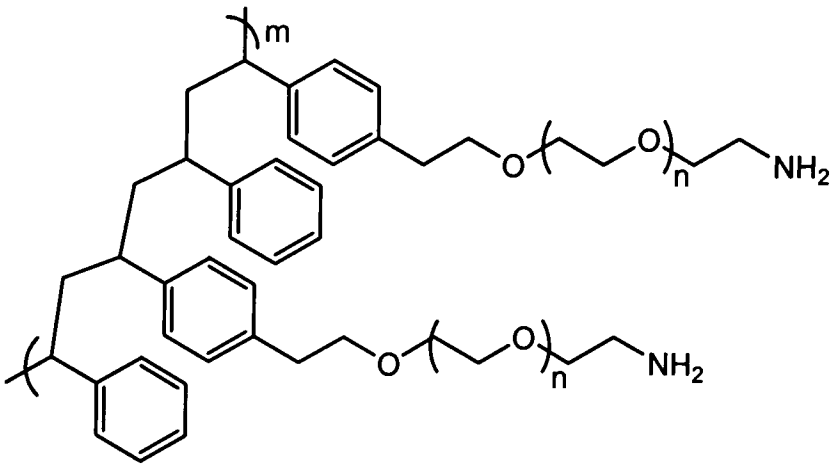
Amino-methyl polystyrene: The polystyrene resin was based on copoly(styrene-1% divinylbenzene).

Amino-Tentagel: The Novasyn® TentaGel resin was based on a composite of low-cross linked polystyrene and polyethylene glycol which had been terminally functionalized with amino groups.

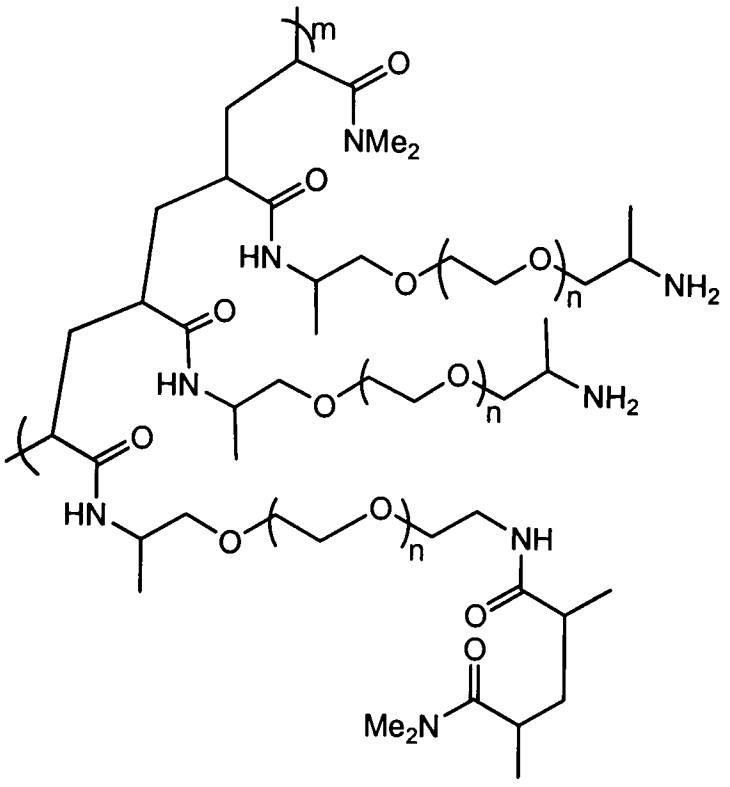
$PEGA_{1900}$: Consisted of a *co*-polymer of *bis*-2-acrylamidoprop-1-yl polyethyleneglycol$_{1900}$.



Amino-methyl polystyrene



Amino-TentaGel



Amino-PEGA

Contents

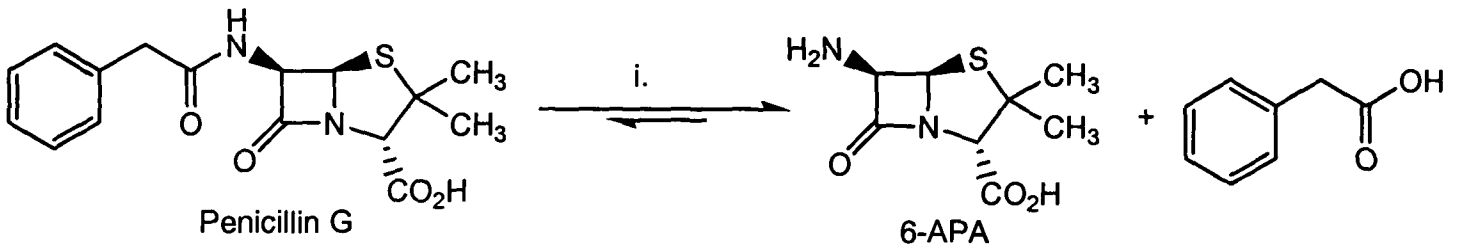
1 Introduction

1.1 Hydrolytic enzymes

Enzymatic methods have many advantages over classical chemical techniques because enzyme catalysed transformations proceed under very mild conditions (pH5-8, 25-37 °C) and with striking chemo-, regio-, and stereoselectivity. Hydrolytic enzymes catalyse the cleavage of C-O, C-N and C-C bonds and some other bonds including phosphoric anhydride bonds.[1] Hydrolytic enzymes are the most useful enzymes for synthesis because of their versatility and ease of use.

1.1.1 Role and action of Penicillin acylase

Penicillin acylase (EC 3.5.1.11) from *Escherichia coli* (ATCC 9637) is a serine type enzyme, which catalyses the hydrolysis of penicillin G to 6-aminopenicillanic acid (6-APA) and phenylacetic acid (Scheme 2).

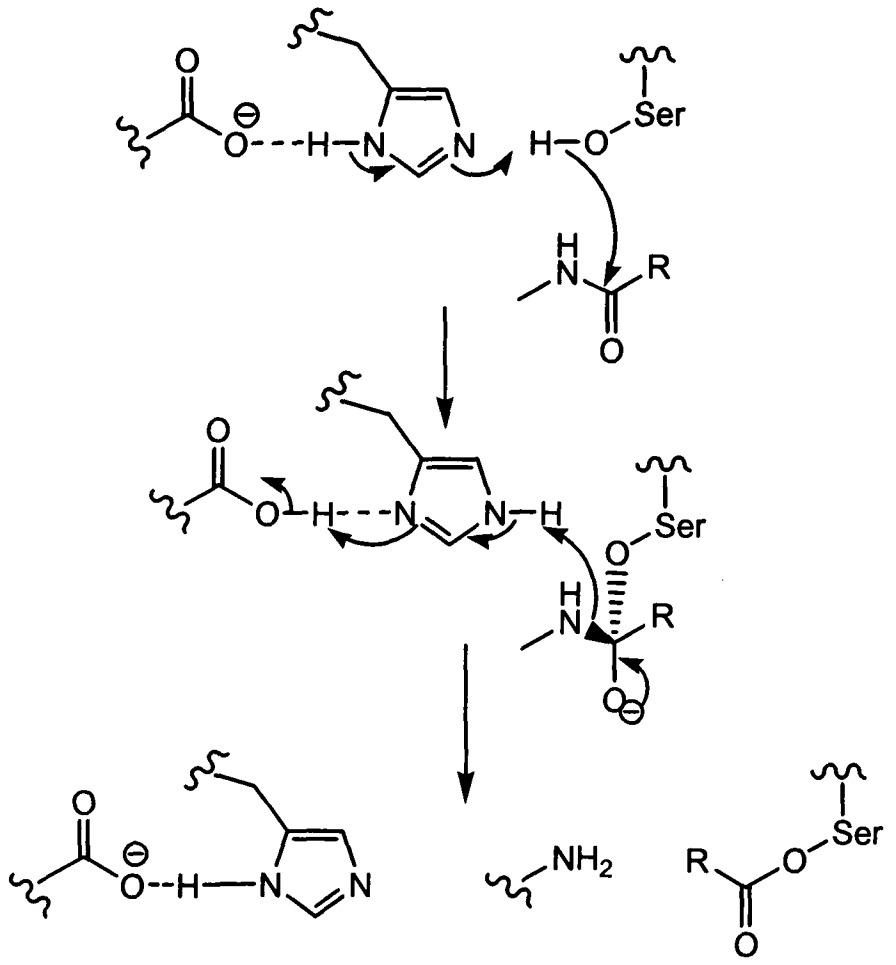


Scheme 2: *Reagents and conditions:* i. Penicillin G acylase.

Penicillin acylase is a heterodimeric protein consisting of a small α subunit and a large β subunit. The catalytic nucleophile, a serine, is found at the *N*-terminus, which is characteristic of the family of *N*-terminal-nucleophilic hydrolases. The catalytic domain is constructed exclusively from the β chain[2] and is situated at the base of a large cavity whose walls are mostly formed by the β chain. This cavity contains a hydrophobic pocket which is lined with a number of aromatic residues and hydrophobic side chains. This renders the enzyme highly specific for the phenylacetyl group of penicillin G. Figure 1 shows the structure of penicillin acylase with penicillin G in the active site.

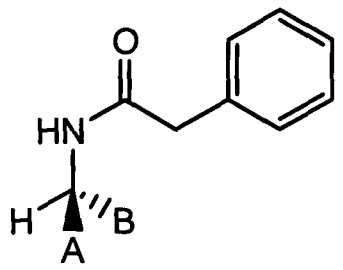
Figure 1: *Structure of Penicillin acylase with penicillin G in the active site.*[3]

The mechanism of hydrolysis is based on a 'catalytic triad' composed of aspartate, histidine and serine (Scheme 3). Initially, a covalent *O*-acyl intermediate is formed, which is de-acetylated with water in the second step.



Scheme 3: *Mechanism of penicillin acylase catalysed formation of O-acyl intermediate.*

Penicillin acylase has been successfully employed in organic synthesis for the cleavage of *N*- or *O*-phenylacetyl protecting group from amino acids, peptides, amines, alcohols and sugars.[4,5] In general, the enzyme accepts substrates with stereo-structures related to L-amino acids[1] (Figure 2).



A = $CONH_2$, CO_2R, CH_2CO_2H, $(CH_2)_nCO_2H$ (n = 0-2)
B = alkyl groups

Figure 2: ***Preferred stereoisomer for penicillin acylase hydrolysis.***

1.1.2 Role and action of lipases and hemiaminalases

Lipases and esterases are serine hydrolases that catalyse the hydrolysis and synthesis of water insoluble long chain triglycerides and small partially water soluble esters, respectively. The active site of lipases is characterised by a triad made up of

serine, histidine and aspartate/glutamate[6] and is similar to that found in serine proteases (Scheme 3). Unlike esterases, lipases display little activity in aqueous solutions with soluble substrates. A sharp increase in activity is observed when the substrate concentration is increased beyond its critical micellular concentration (Figure 3). This event was termed 'interfacial activation'[7].

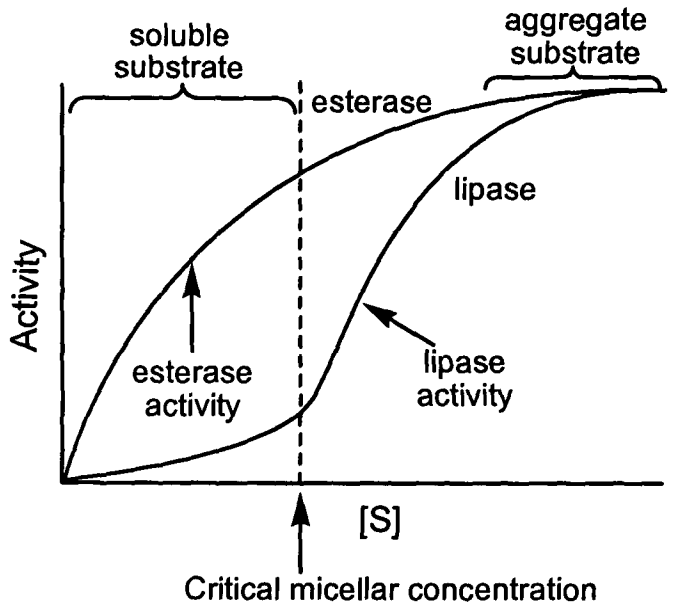
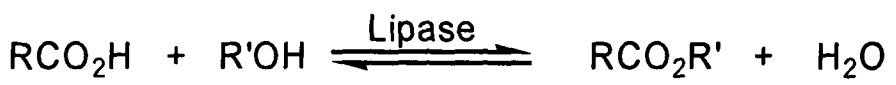


Figure 3: *Interfacial activation of lipases.*

In addition, most lipases possess a helical oligopeptide unit that shields the active site, termed a 'lid'. This 'lid'[8], upon contact with a hydrophobic interface, moves away to expose the active site providing access for the substrate. It should be noted however, that not all lipases contain a 'lid' or show interfacial activation.[6]

1.1.3 The use of lipases/esterases in organic synthesis

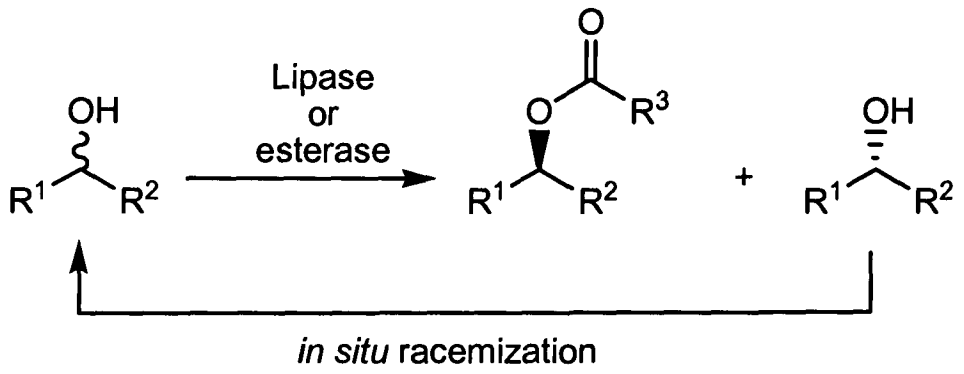
Lipases and esterases are the most commonly used enzymes in synthetic organic chemistry, catalyzing the chemo-, regio- and/or stereoselective hydrolysis of carboxylic acid esters or the reverse reaction in organic solvents[6,9,10] (Scheme 4).



Scheme 4: *Reversible lipase/esterase catalysed reactions.*

Lipases and esterases have been widely applied to the preparation of enantiopure compounds from racemic mixtures, prochiral or *meso*-compounds, or

diastereomeric mixtures.[6] In kinetic resolutions, theoretical yields are limited to 50% because the enzyme only hydrolyses one enantiomer of the racemic starting material. This limits the applicability of enzymes to organic synthesis; however, the advent of dynamic kinetic resolution has overcome this problem. Dynamic kinetic resolution involves kinetic resolution with *in situ* racemization (Scheme 5).



Scheme 5: *Dynamic kinetic resolution.*[1]

In addition, lipases/esterases have been utilized for the mild, selective removal of protecting groups from amines[11] and alcohols *etc.*[12] These are described in several reviews.[4,5,13]

1.2 Biocatalysis in polymer-supported synthesis/hydrolysis

In classical organic synthesis, enzymes have been used for many years for a wide variety of chemical transformations, yet their application to solid phase synthesis has been limited.[14] In order for a reaction to occur in polymer matrices, swelling and diffusion of solvents and reagents into the polymer matrix needs to prevail, before a reaction can take place. It is now accepted that polymer supports should be treated as solvents in which the reactions are performed[15], rather than as inert solid supports. Therefore, the choice of polymer support is crucial to the success of enzyme hydrolysis on solid support. Enzyme hydrolysis is usually performed in aqueous media, which considerably limits the suitability of the available solid supports. The types of resin suitable for enzymatic transformations fall into three broad categories:

- Swelling resins that allow penetration by the enzyme.
- Non-swelling resins where reaction occurs at a functionalized surface.

- Soluble resins where the enzymatic transformation occurs in solution and the resin is precipitated at the end of the reaction.

Enzyme catalysis on solid supported substrates is attracting interest for various applications: *e.g.* chemo-enzymatic synthesis on solid supports,[16,17] 'on-bead' screening for enzyme substrates or inhibitors,[18] enzyme cleavable protecting groups in solid phase chemistry[4] and enzyme cleavable linkers for release of polymer bound substrates.[19] Several reviews have been published that deal with polymer supported enzyme transformations; although, these focus on enzyme cleavable linkers in polymer supported synthesis.[14,19,20] The purpose of the following review is to provide a more complete picture of the literature, covering general polymer-supported enzymatic transformations.

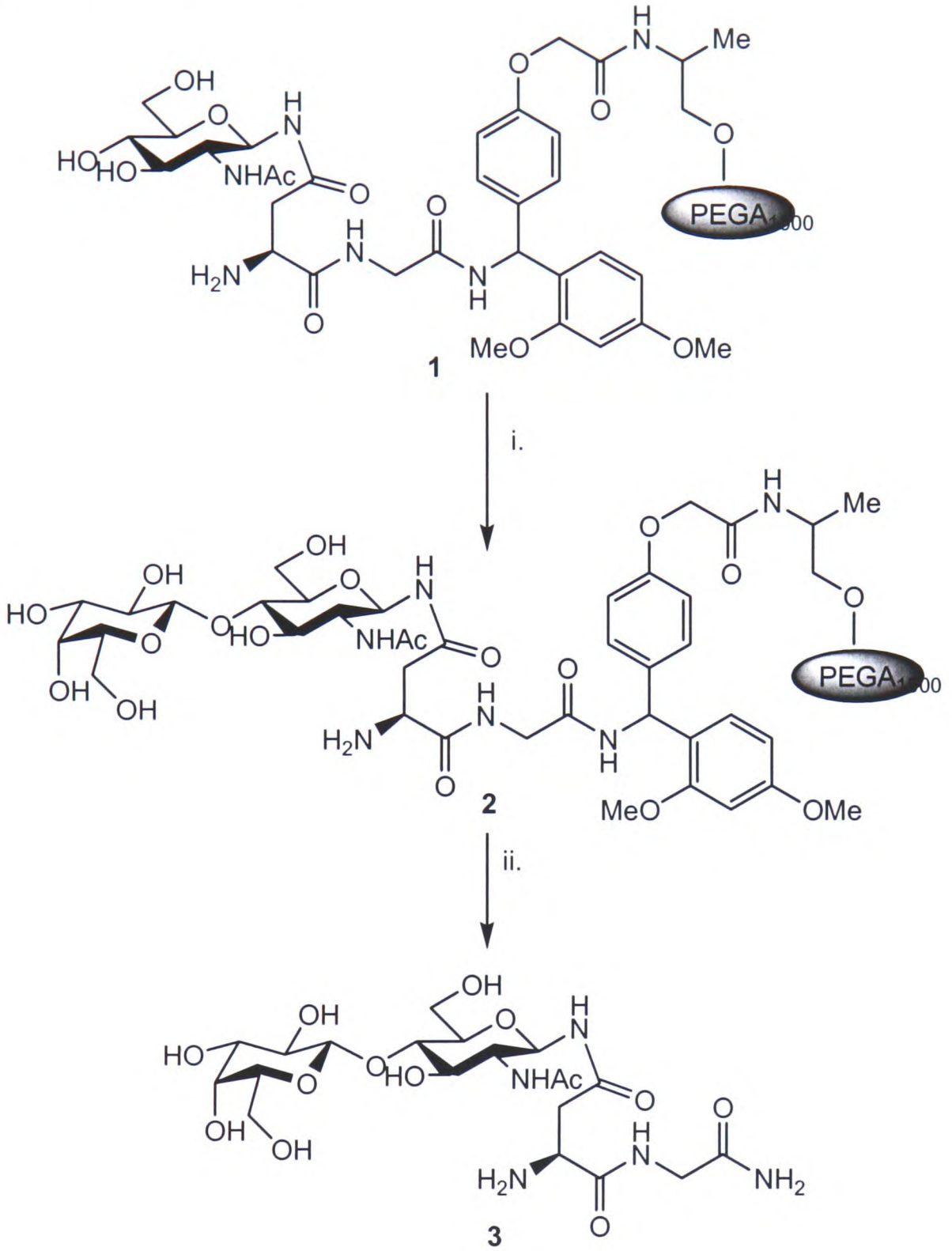
1.2.1 Chemo-enzymatic synthesis on solid supports

1.2.1.1 Swelling resins

The first application of beaded polyethylene glycol polyacrylamide copolymer ($PEGA_{1900}$) as a solid support for the chemo-enzymatic synthesis of glycopeptides was reported by Meldal *et. al.*[17] (Scheme 6). The glycopeptide, was anchored to $PEGA_{1900}$ *via* an acid labile linker **1**. Treatment of **1**, with bovine β-(1,4)-galactosyltransferase (50 kDa) and UDP-Gal for 48 hours, afforded glycopeptide **2** in 95% yield, after acid cleavage. It was discovered that pre-incubation of **1** with enzyme and buffer for 3 days at 4 °C before addition of UDP-Gal gave >90% conversion in 6 hours. Therefore, it was proposed that the extended reaction time (48 h) was probably needed for the polyethylene glycol (PEG)-polymer to refold around the protein, allowing it to move around inside the gel-like resin and access all the reaction sites.

Similar synthesis experiments carried out using a more highly crosslinked $PEGA_{1900/300}$ resin resulted in only 50% conversion of **1** after 72 hours. This

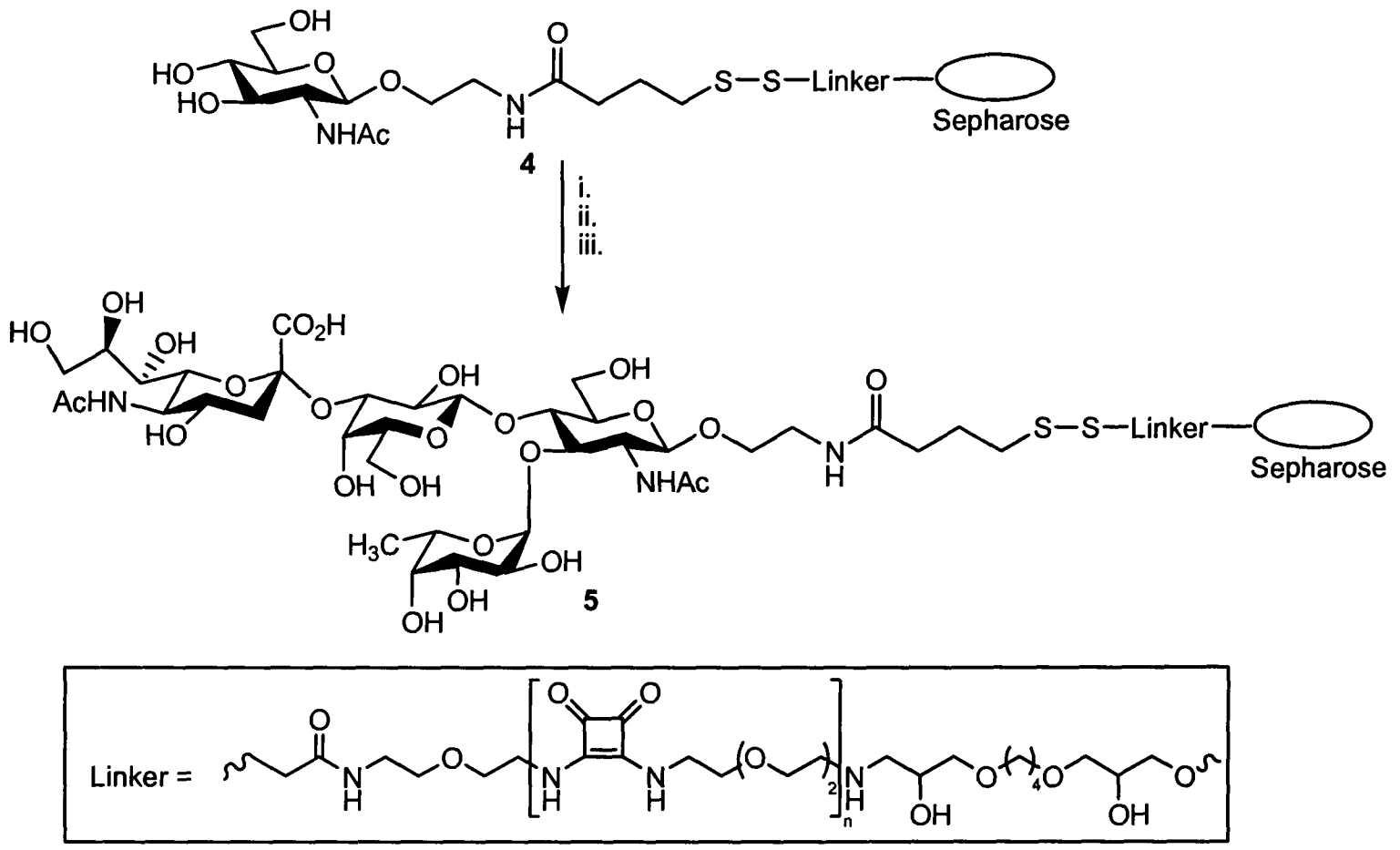
illustrates the importance of selecting the correct level of crosslinking in polymers for biocatalytic transformations.



Scheme 6: *Reagents and conditions:* i. bovine β–(1,4)glycosyltransferase, UDP-Gal, buffer pH 7.4, 23 °C, 48 h, 95%; ii. 95% TFA/H_2O, 23 °C, 2 h.

In 1998, Blixt and Norberg[21] utilized a sepharose 6B matrix for the enzymatic synthesis of a sialyl Lewis X tetrasaccharide **5** (Scheme 7). Sepharose 6B is an agarose based gel matrix which swells in aqueous media and is permeable to large biomolecules. One drawback is that sepharose 6B is unstable in organic solvents, and thus, it is unsuitable for chemical synthesis. Enzymatic glycosylation of **4** with galactosyl-, sialyl- and fucosyltransferase afforded sepharose bound tetrasaccharide

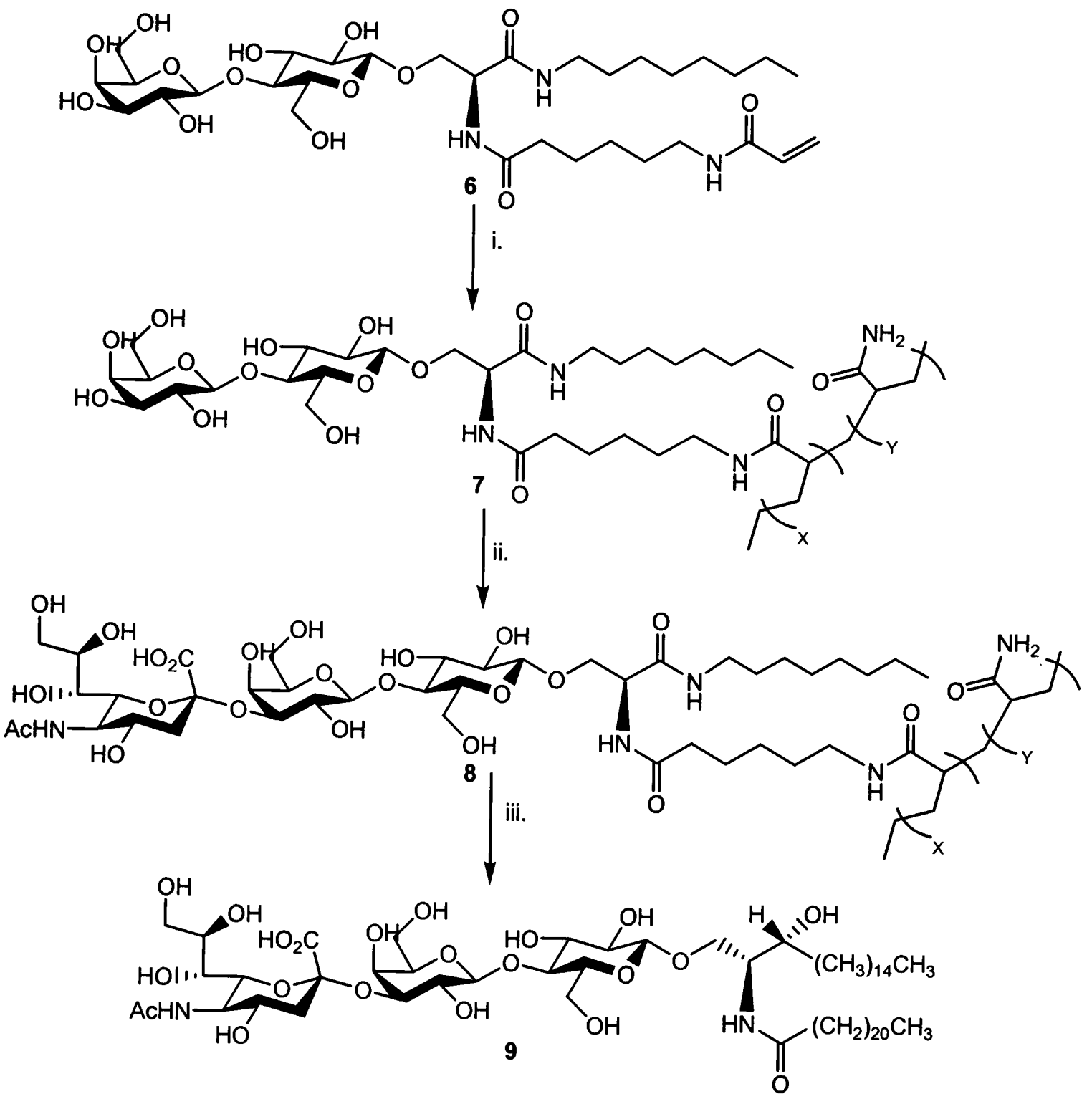
5, after cleavage from Sepharose with dithiothreitol (DTT) (in 57% overall yield). Linkers of various lengths, n, were tested to optimize the galactosylation with galactosyltransferase. A clear correlation between linker length, n, and yield was found in the galactosylation of **4** (*i.e.* n = 0 (70%); n = 3 (98%)).



Scheme 7: *Reagents and conditions:* i. cacodylate, $MnCl_2$, TritonX, pH 7.5, β-1,4-galactosyltransferase, alkaline phosphatase, α-lactalalbumin, UDP-Gal, 37 °C, 5 d, when n = 3, 98%; ii. buffer pH 7.45, α-2,3-sialyltransferase, CMP-Neu5Ac, alkaline phosphatase, 5 d, when n = 3, 75%; iii. α-1-(3/4)-fucosyltransferase, buffer pH 6.5, GDP-fucose, alkaline phosphatase, 5 d, when n = 3, 95%.

1.2.1.2 Soluble polymers

Nishimura and Yamada[22] introduced the use of ceramide glycanase for the synthesis of ganglioside GM3, **9** (Scheme 8). Radical polymerization of **6**, generated the water-soluble polyacrylamide bound glycopolymer **7**. Ganglioside **9**, was synthesized by sialylation of **7** with sialyltransferase, followed by transglycosylation of **8** with ceramide glycanase in the presence of ceramide as acceptor (61% yield).

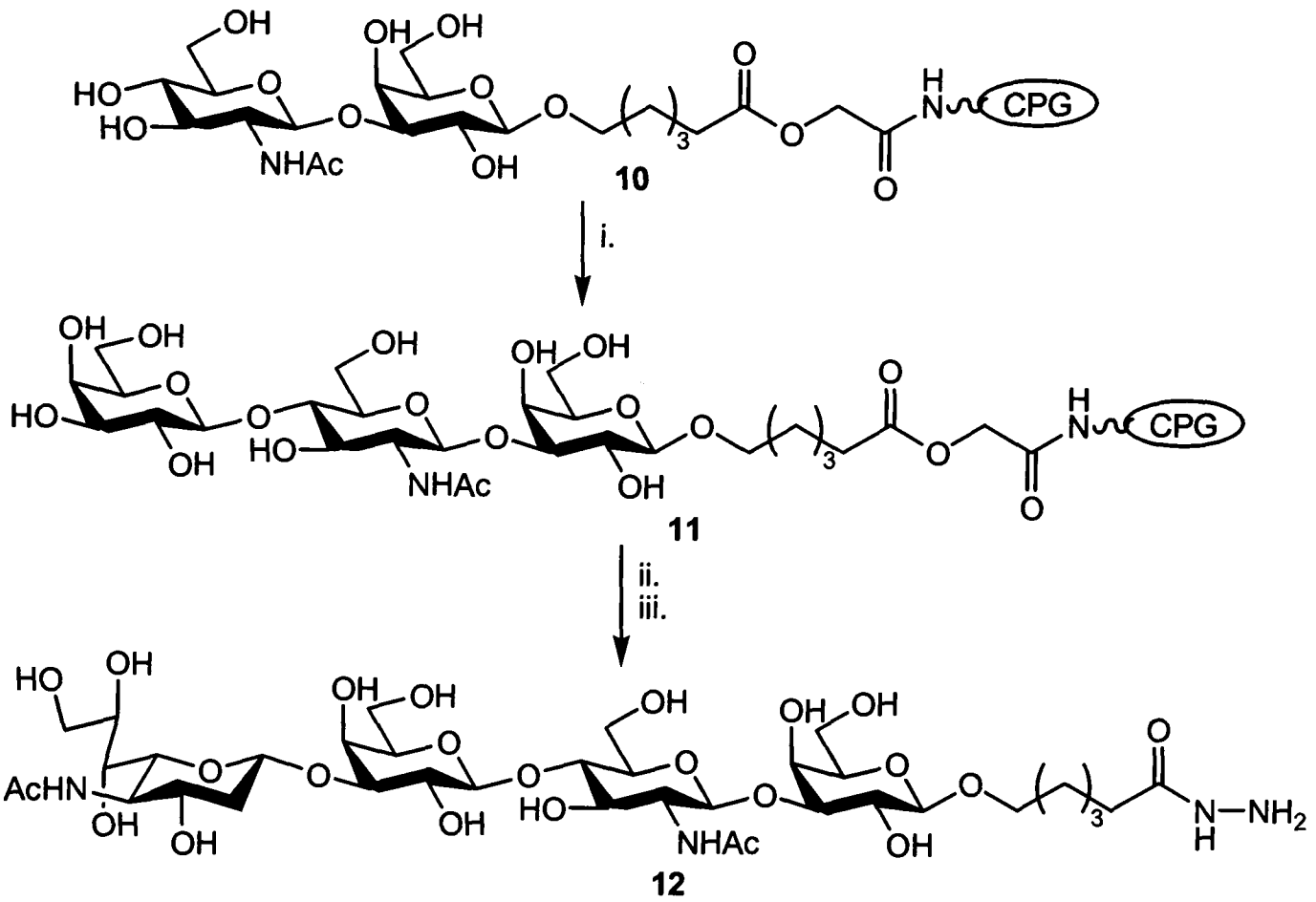


Scheme 8: *Reagents and conditions:* i. acrylamide, TEMED, ammonium peroxodisulfate, DMSO-H_2O, 50 °C, 48 h; ii. CMP-NeuAc, sialyltransferase, BSA, $MnCl_2$, CIAP, sodium cacodylate buffer (pH 7.5), 37 °C, 3d; iii. Ceramide, ceramide glycanase, Triton CF-54, sodium citrate buffer (pH 6), 37 °C, 17 h, 61%.

1.2.1.3 Non-swelling resins

Halcomb *et. al.*[23] have utilized glycosyltransferases for the solid phase synthesis of a tetrasaccharide, 12 (Scheme 9). A disaccharide primer was attached to controlled pore glass *via* an ester spacer group to give **10**. Enzymatic glycosylation was carried out with UDP-Gal and β-1,4-galactosyltransferase to afford trisaccharide **11**, which was sialylated using α-sialyltransferase. Subsequent cleavage with

hydrazine hydrate gave tetrasaccharide **12** in >98% yield. No details were given about the controlled pore glass support, such as pore size *etc.*



Scheme 9: *Reagents and conditions:* i. β-(1,4)-galactosyltransferase, UDP-Gal, DTT, HEPES buffer pH 7.2, $MnCl_2$, 48 h; ii. α-sialyltransferase, CMP-NeuAc, DTT, Triton-X, alkaline phosphatase, BSA, buffer pH 7.5, 48 h; iii. hydrazine monohydrate, r.t., 24 h.

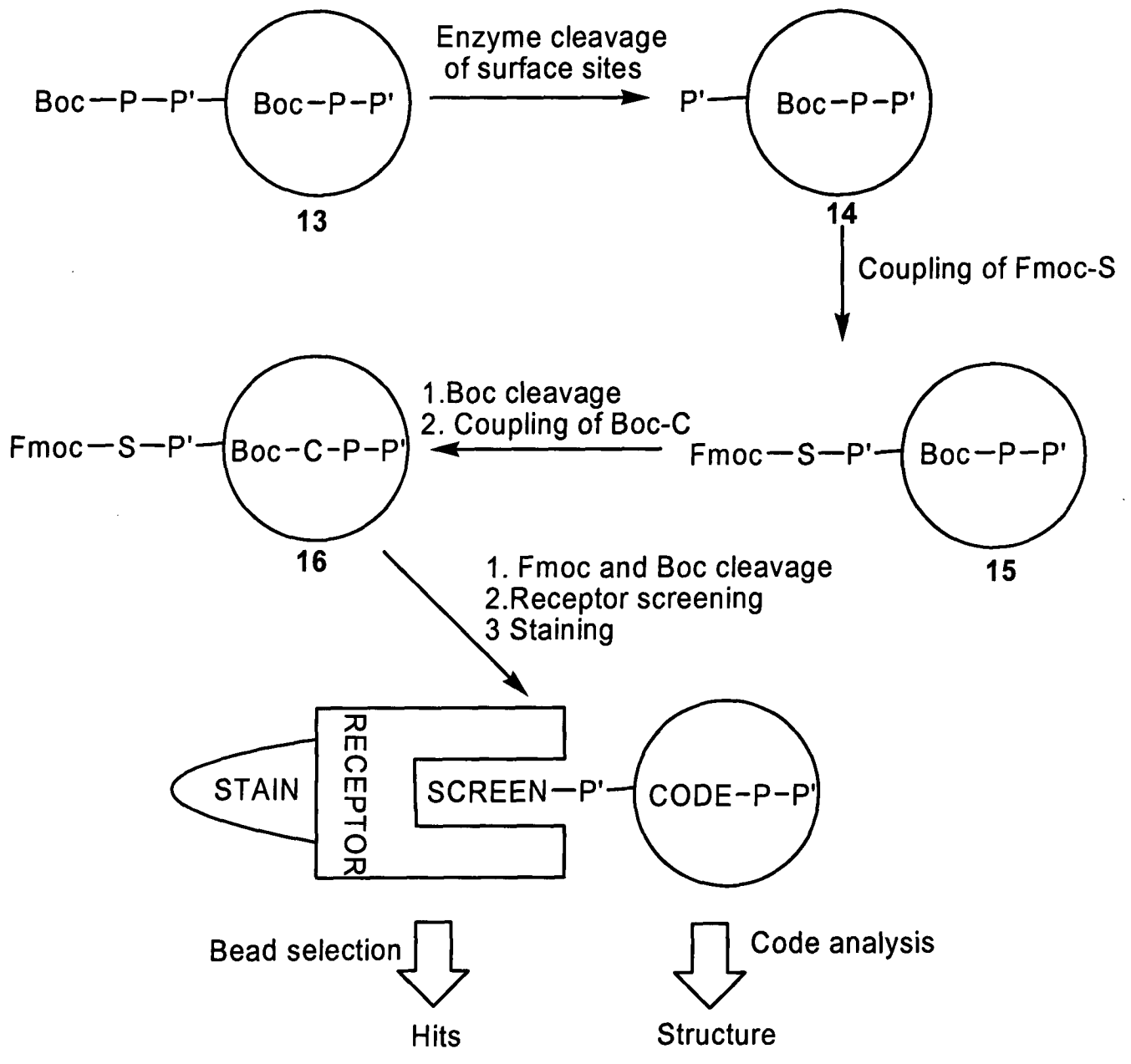
1.2.2 'On bead' screening for enzyme substrates or inhibitors

Resins suitable for solid-phase enzymatic synthesis are often also useful for the solid-phase screening of enzyme substrates/inhibitors.

1.2.2.1 Swelling resins

The limited accessibility of enzymes to the interior of polymer beads was exploited in the generation and screening of encoded combinatorial peptide libraries. Barany *et. al.*[24] used the different physical characteristics of the 'surface' and 'interior' of TentaGel resin to selectively cleave short *N*-protected peptide substrates with chymotrypsin (22 kDa), from the surface area only. ('shaving') (Scheme 10). The interior sites were left uncleaved to give **14**. Chymotrypsin shaving saturated at 2-2.5% of the total sites, whereas elastase or pepsin access 10-15% of the sites. It

was also found that increased exposure to the enzyme did not increase the amount shaved. Fmoc-chemistry was utilized to attach the first residue **15**. Subsequent elaboration of the interior sites using orthogonal Boc chemistry generated the code structure. Repetition of this process furnished a surface peptide, which was encoded internally **16**. Biological screening was carried out with different receptors (anti-β-endorphin antibody (170 kDa), streptavidin (60 kDa) and thrombin (35 kDa)) to reveal the active species on the surface areas, and sequencing of the beads thus identified gave the corresponding interior coding structures. These coding structures were used to deduce the binding structures of the surface active species. This screening method led to the discovery of a new thrombin ligand Arg-Gly-Arg-Pro-D-Phe which binds with an order of magnitude greater affinity than the natural motif D-Phe-Pro-Arg-Pro-Gly.



Scheme 10: *'Shaving' experiments on TentaGel resin.*

Bradley *et. al.*[25] performed experiments to compare resin and solution screening methodologies in combinatorial chemistry. This was achieved by the preparation of three identical polyamine peptide libraries; one in solution and the other two were resin-based (PEGA **17** and TentaGel **18**) (Figure 4). All three libraries were assayed for inhibition of the enzyme, trypanothione reductase (55 kDa). In the case of the TentaGel-based library **18**, no reduction in activity of the enzyme was observed. However, screening of PEGA resin library **17** was successful and inhibitors of trypanothione reductase were identified. Most importantly, there was good agreement between the solution and resin-based assays.

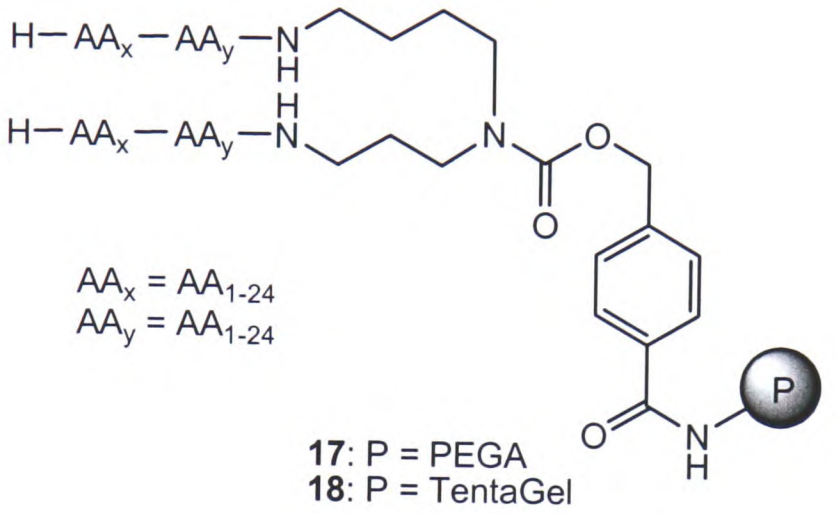


Figure 4: *Polyamine libraries on PEGA 17 and TentaGel 18 resins.*

In 1994, Meldal *et. al.*[26] described a solid phase assay for the complete subsite mapping of the active site of endoproteases. Kieselguhr-supported polydimethylacrylamide and PEGA resins were used as solid supports for the synthesis of a library of protease substrates. *O*-aminobenzamide (Abz) and 3-nitrotyrosine ($Y(NO_2)$) were used as donor-acceptor pairs for the visualisation of internally quenched substrates by fluorescence resonance energy transfer (FRET) analysis. The Abz group can be observed visually in the absence of 3-nitrotyrosine, whereas peptides containing both donor and acceptor are quenched and appear dark under a fluorescence microscope. Peptide synthesis was performed and the permeability of the resin was studied using subtilisin Carlsberg (27 kDa) on the model substrate, [$Y(NO_2)$FQPLDEK(Abz)] bound to PEGA resin **19** (Figure 5). It was found that upon addition of the enzyme to the beads containing the model substrate **19**, a clear blue ring formed rapidly at the periphery of substrate-containing beads, **20**. Within 1 hour, the beads were completely illuminated and peptide

sequencing showed the substrate to be 80% cleaved, **21**. After 24 hours, the cleavage was quantitative, **22** indicating the complete permeability of the PEGA resin. The PEGA resin contained a high content of polyethylene glycol, however the molecular weight of the resin was not specified.

Similar results were obtained with the kieselguhr-supported model substrate.[26] This assay allowed for determination of the substrate preferences for subtilisin Carlsberg and the result was compared with data from kinetic studies in solution. Although there were discrepancies between the assay on solid phase and in solution, which may be due to the differences in the micro-environment and dynamic properties of the substrates; the ranking of substrates in solution and solid phase was identical. Therefore, the influence of the solid support on the substrate preference of the enzyme was small enough to allow for the design of substrates using this assay.

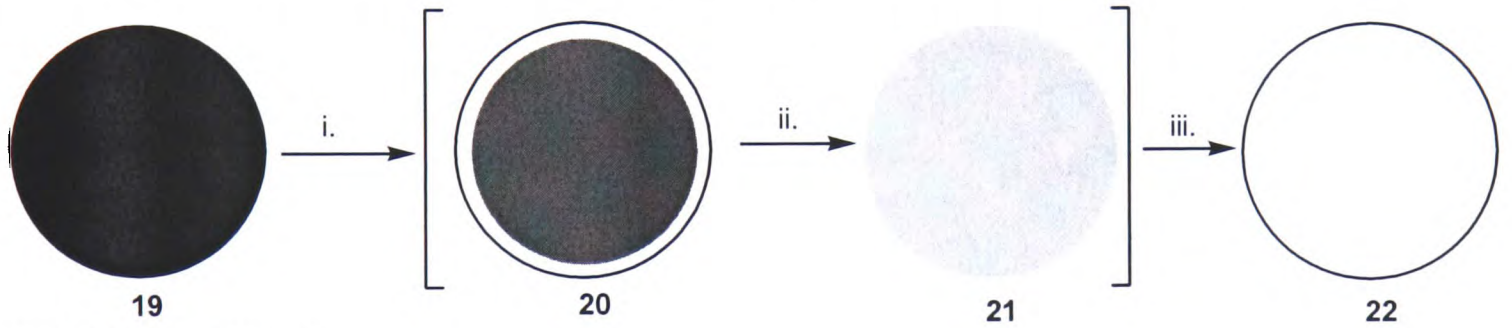
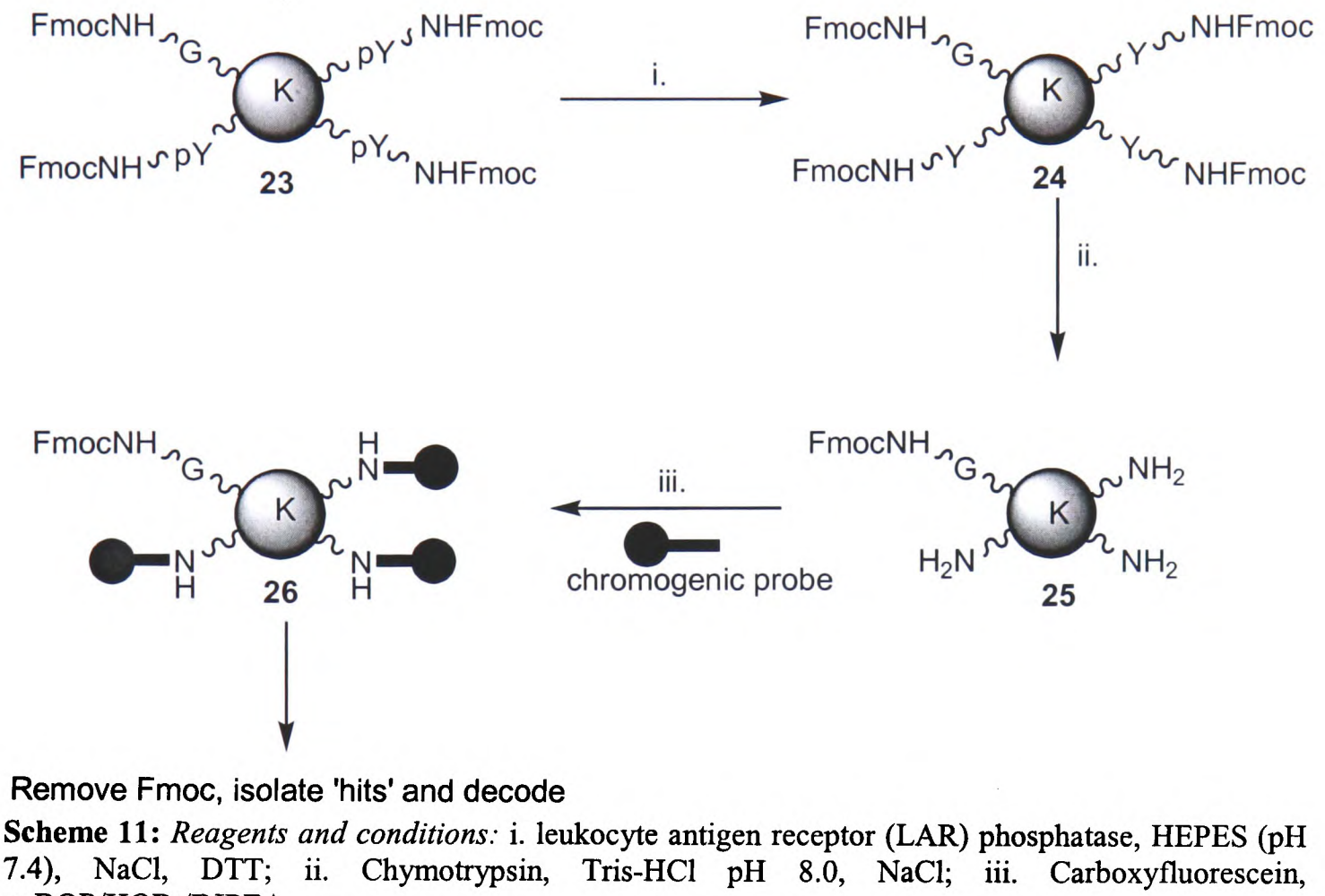


Figure 5: *Visualisation of PEGA resin under a fluorescence microscope after treatment with subtilisin Carlsberg;* i. addition of subtilisin Carlsberg; ii. 2 h later (80% cleaved); iii. 24 h later (quantitative cleavage).

Meares *et. al.*[27] have also successfully employed the internally quenched fluorescence donor/acceptor pairs (Abz/Y(NO_2) for the FRET-based visualisation of substrates for endoproteases, cathepsin B and D (52 and 56 kDa), bound to $PEGA_{800}$ resin.

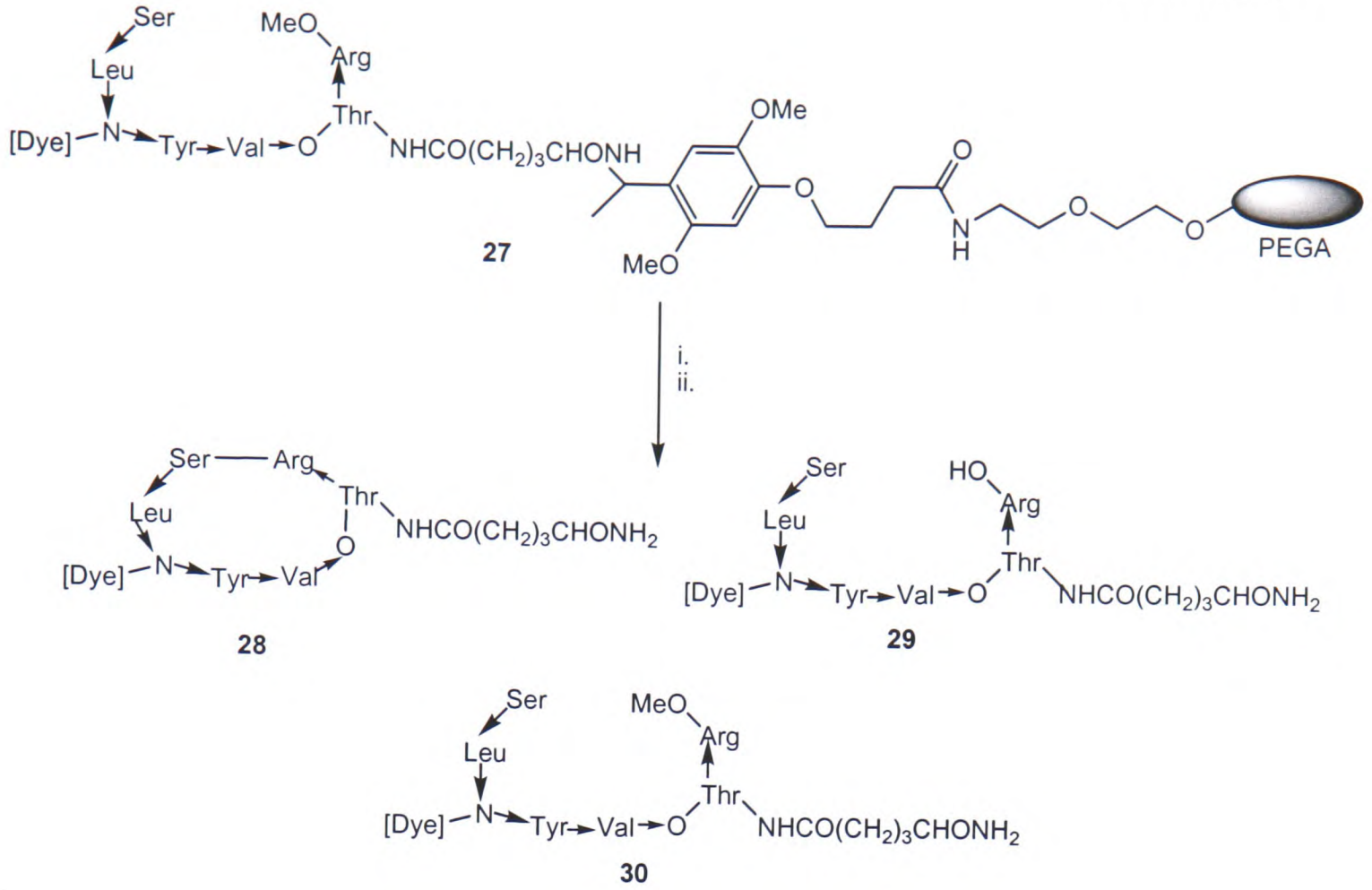
Balasubramanian *et. al.*[28] have developed a combinatorial screening method for identifying substrates for protein tyrosine phosphatases (PTP) (35 kDa) (Scheme 11). Phosphotyrosyl peptides were synthesised on amino-functionalised resin using Fmoc chemistry. Near quantitative dephosphorylation of the resin bound peptide **23** was achieved on Kieselguhr and PEGA resin with leukocyte antigen receptor (LAR) PTP, compared to less than 2% with TentaGel resin. Gel filtration studies showed that the size exclusion limit of TentaGel was below 14 kDa. The screening strategy is

illustrated in Scheme 11. A phosphopeptide library is synthesized in which the phosphotyrosyl (pY) position was doped with glycine to form the encoding strand. Dephosphorylation was carried out by incubation with LAR PTP to afford tyrosine peptides **24**. Subsequent treatment with α-chymotrypsin, selectively cleaved the dephosphorylated peptides. Coupling of a chromogenic probe, such as carboxyfluorescein to **25** allowed for isolation of the active substrate **26** which was identified by peptide sequencing of the encoding strand.



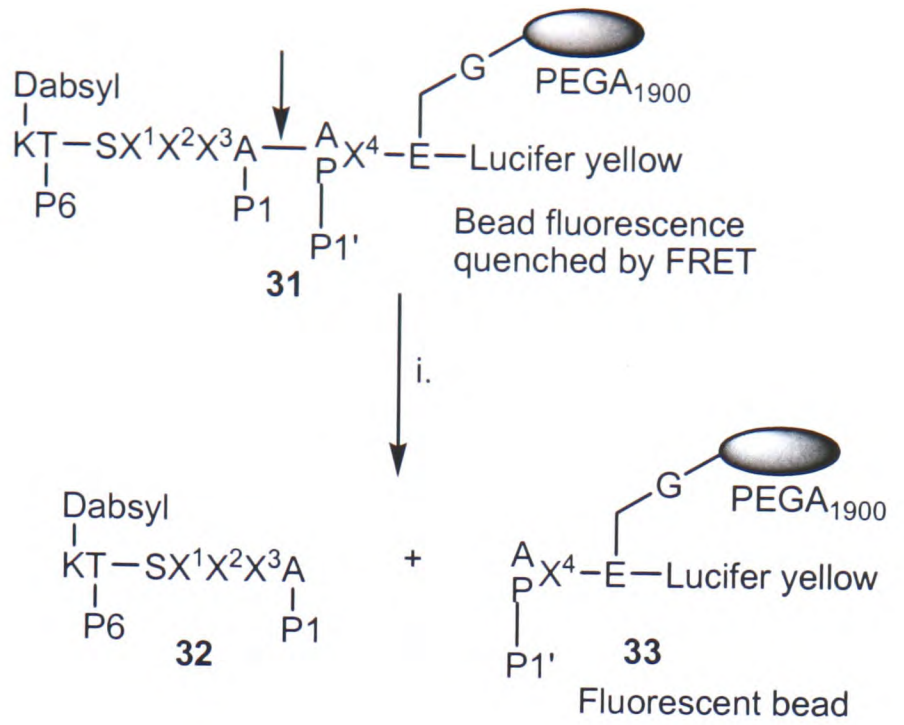
Scheme 11: *Reagents and conditions:* i. leukocyte antigen receptor (LAR) phosphatase, HEPES (pH 7.4), NaCl, DTT; ii. Chymotrypsin, Tris-HCl pH 8.0, NaCl; iii. Carboxyfluorescein, pyBOP/HOBt/DIPEA.

Burger and Bartlett[29] have synthesized an analogue of trypsin inhibitor A90720A, **28** by trypsin mediated macrocyclisation on PEGA resin **27** (Scheme 12). Analysis of the product mixture revealed a 1:1 mixture of cyclised product **28** to hydrolysis product **29**. Only a small amount of precursor **30** was obtained, indicating that most of the material on **27** was accessible to the enzyme. When similar enzymatic cyclisations were attempted with TentaGel resin, no products from enzymatic transformation were detected.



Scheme 12: *Reagents and conditions:* i. trypsin, 30:30:40 DMF/ethanol/pH 6.5 Tris buffer; ii. Photolysis.

Fluorescence resonance energy transfer (FRET) analysis has also been employed in the screening of peptide libraries for the identification of substrates for proteolytic enzymes, *E. coli* Leader peptidase (37 kDa) and Napsin A (38 kDa)[30] (Scheme 13). Lucifer yellow was used as the fluorescent donor and Dabsyl, as fluorescent quenching acceptor. The peptide library was synthesized on $PEGA_{1900}$ resin. When the beads **31** are incubated with a protease, the enzyme cleaves its preferred substrate sequence and liberates the quencher **32** from those peptides into solution. The beads carrying a cleaved substrate **33** become fluorescent and can be detected under a fluorescent microscope among the quenched and therefore dark beads. The fluorescent beads **33** are collected and sequenced by Edman degradation.

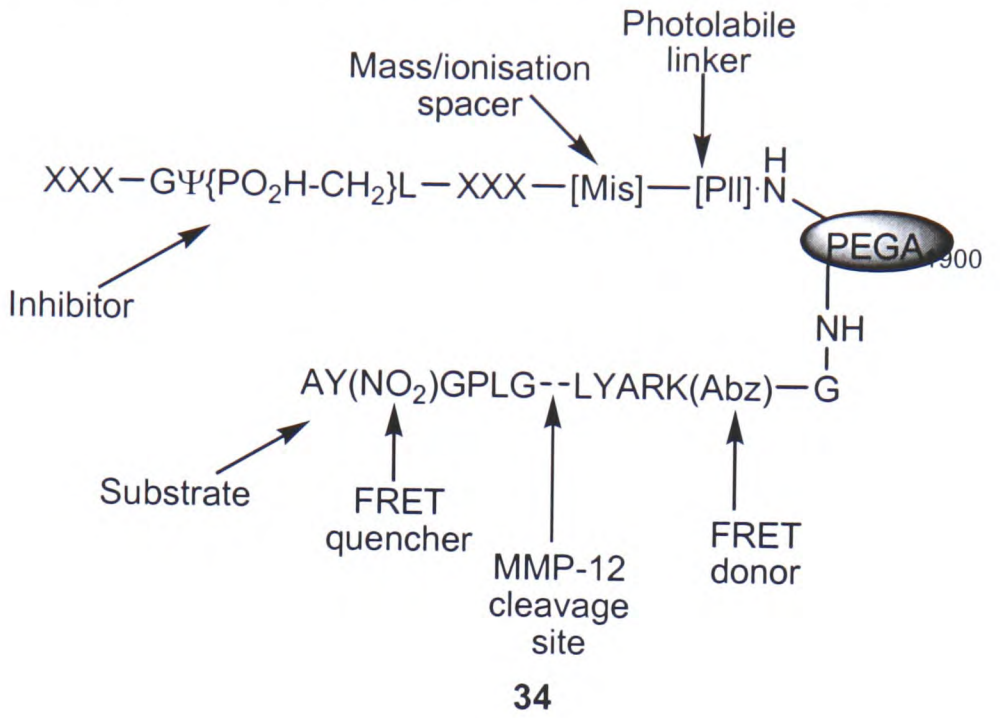


Scheme 13: *Reagents and conditions:* i. *E. coli* leader peptidase, Tris-HCl buffer pH 7.4, $MgCl_2$, Triton X-100, glycerol. *Black arrow indicates site of cleavage. X represents all possible combinations of the 19 natural amino acids. Abbreviations for the amino acid residues are as follows: K = Lys, T = Thr, A = Ala, S = Ser, E = Glu, G = Gly, P = Pro.*

Meldal *et. al.*[31] have described the synthesis of high molecular weight $PEGA_{6000}$ resins and their use as solid supports for identification of substrates for matrix metalloproteinase (MMP-9, 67/83 kDa). $PEGA_{6000}$ bound peptide libraries (general formula; $XXY(NO_2)XXXXXXK(Abz)PPPM\text{-}PEGA_{6000}$, where X represents 20 amino acids except cysteine) were synthesized with *o*-aminobenzamide (Abz) and 3-nitrotryrosine as the fluorescent donor-acceptor pair for visualization by fluorescence microscopy. Treatment of the resin bound peptide libraries with MMP-9 at 37 °C for 40 h resulted in fluorescent beads which could be separated from the non-fluorescent beads and subsequent peptide sequencing carried out to determine the preferred substrate sequences for MMP-9.

Buchardt *et. al.*[32] have screened a solid phase library of 165,000 phosphinic peptide inhibitors for activity against MMP-12. The library was assembled on $PEGA_{1900}$ resin as a one-bead-two compound library **34** and every bead contained a common quenched fluorogenic substrate and a different inhibitor (Figure 6). When MMP-12 (22 kDa) enters one of these beads, competition occurs between the substrate and inhibitor: the stronger the inhibitor present on a specific bead, the less

substrate is cleaved. A bead containing a poor inhibitor would allow the enzyme to cleave the quenched fluorogenic substrate and the bead would light up when viewed under a fluorescence microscope. Therefore, the darkest beads were selected and irradiated with a Hg lamp, which released the inhibitor from the photolabile linker. MALDI-TOF mass spectrometry, afforded the sequence of the active compound.



34

Figure 6: *Structure of the one-bead-two compounds solid phase library* ***34*** *for the screening of phosphinic peptide inhibitors of MMP-12. X represents arbitrary α-amino acids.*

Meldal *et. al.*[33,34] have also used one-bead-two compound fluorescent inhibitor assays, for the successful identification of inhibitors of cysteine proteases from *Leishmania mexicana* CPB2.8ΔCTE and cruzipain (57kDa). In the first paper,[33] the inhibitor assay was bound to $PEGA_{4000}$ resin and in the second paper,[34] $PEGA_{1900}$ resin was used as the solid support. It was found that cruzipain could not penetrate into the interior of the $PEGA_{1900}$ beads to enable complete cleavage of the substrate from solid support.

In 1998, Leon *et. al.*[35] studied the compatibility of TentaGel, ArgoGel and PEGA, in the screening of papain (23 kDa) activity towards peptide substrates. ArgoGel displays similar characteristics to TentaGel but swells more extensively than TentaGel in aqueous environments, owing to a higher polyethylene glycol (PEG) content. It was therefore expected that ArgoGel might offer greater accessibility to biomolecules. The three sets of beads, **35-37** were treated with papain in pH 6.6 buffer for 1 hour, and examined by fluorescence microscopy. In the

case of PEGA bound peptide **35**, significant reduction in the fluorescence of the beads was observed (Figure 7). Peptide sequencing confirmed near quantitative cleavage of **35**. This indicated that the interior of the PEGA beads was freely accessible to biomolecules such as papain. This is in agreement with the results obtained by Meldal *et. al.*[17,26] In contrast, TentaGel and ArgoGel substrates, **36** and **37**, only afforded low levels of hydrolysis even after prolonged treatment with papain. Additionally, the subsite specificities of papain and chymotrypsin (22 kDa) was determined using PEGA-bound peptide libraries. Unfortunately, no details were given on the molecular weight of PEGA and therefore, the length of PEG chain attached to the resin is not known.

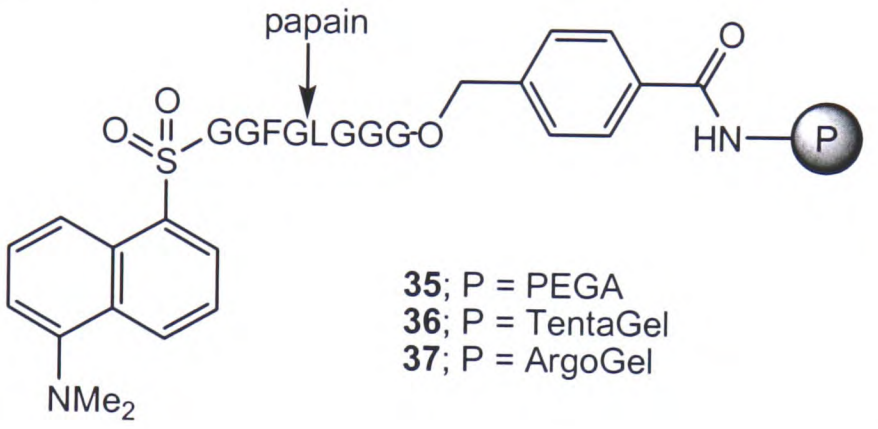
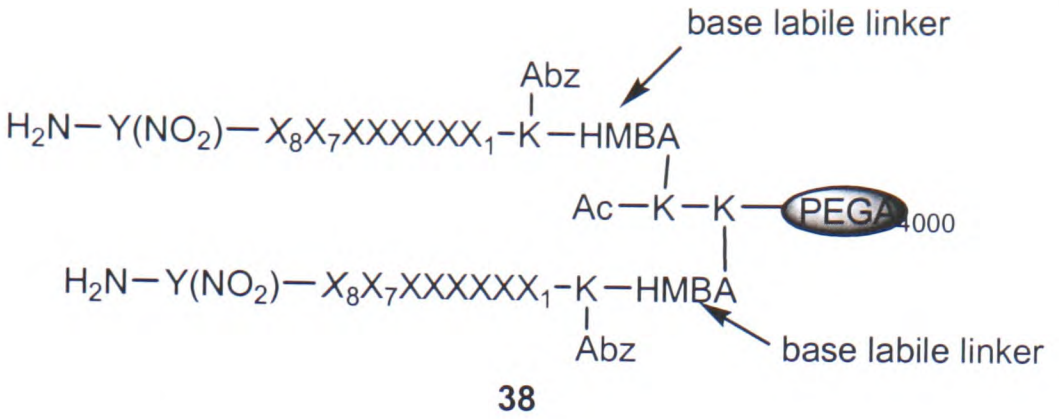


Figure 7: *Resin bound peptide substrates 35-37 labeled with dansyl fluorophore, showing the expected site of cleavage by papain.*

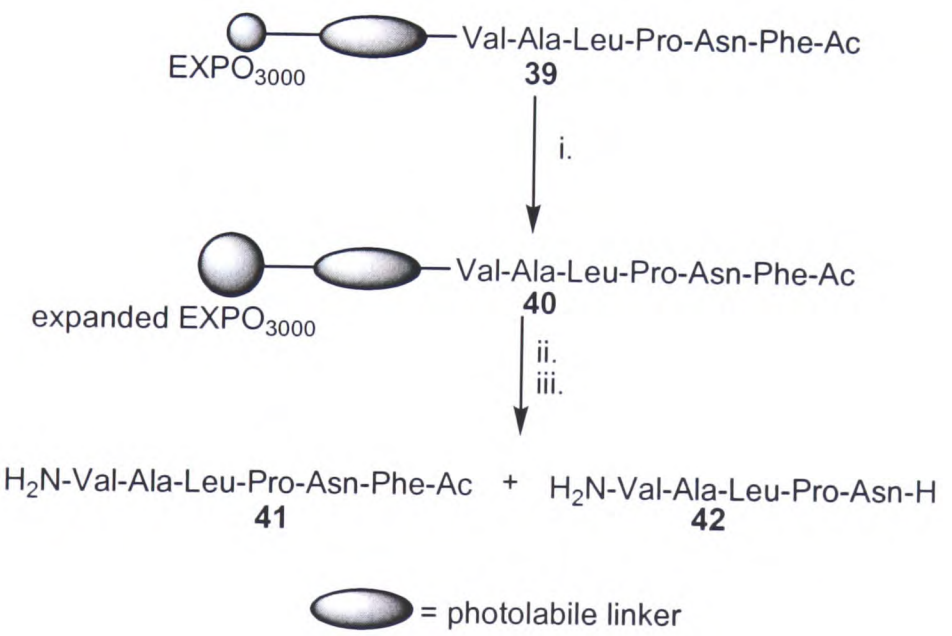
St Hilaire *et. al.*[36] have also used PEGA resin for the mapping of substrate specificities of papain, a cysteine protease. $PEGA_{4000}$ resin was chosen due to its large pore size and proven success with biocatalysts.[31] Internally quenched fluorescent peptide libraries were synthesized with the general structure **38** shown in Figure 8. The libraries were incubated with papain for 2.5 h at 25 °C, and the beads were examined under a UV light to identify fluorescent beads. Subsequent sequencing of the fluorescent beads, by Edman degradation, afforded the peptide sequence. The best substrate was $Y(NO_2)$PMPPLCTSMK(Abz), which afforded 52% cleavage with papain.



38

Figure 8: *General peptide library construct. X_1-X_7 are arbitrary amino acids, X_8 is Pro*

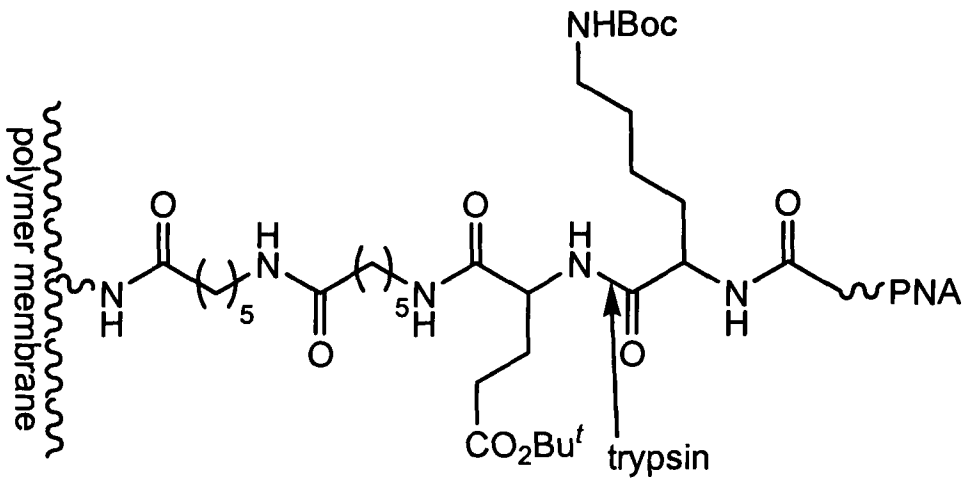
In 2002, Meldal *et. al.*[37] introduced a new polymer, $EXPO_{3000}$, for synthesis and enzymatic assays. The resin is a *co*-polymer of bis-functionalised PEG_{3000} and a highly functionalized hydrophobic silyl crosslinker. Low swelling was achieved in all solvents until cleavage of the silyl based crosslinker on **39**, expands the resin to render it accessible to enzymes (Scheme 14). Treatment of **40** with subtilisin Carlsberg (27 kDa), followed by photolysis afforded a mixture of uncleaved peptide, **41** and hydrolysed peptide, **42**. Unfortunately, no enzymatic cleavage yields were given.



Scheme 14: *Reagents and conditions:* i. TBAF/AcOH, THF; ii. subtilisin Carlsberg, 30 min; iii. hν.

1.2.2.2 Non-swelling resins

Arrays of up to 1000 peptide nucleic acid (PNA) oligomers were generated on polymer membranes **43** by Hoheisel *et. al.*[38] (Figure 9).



43

Figure 9: *Trypsin labile linker **43** for the synthesis of peptide nucleic acid (PNA) oligomer synthesis.*

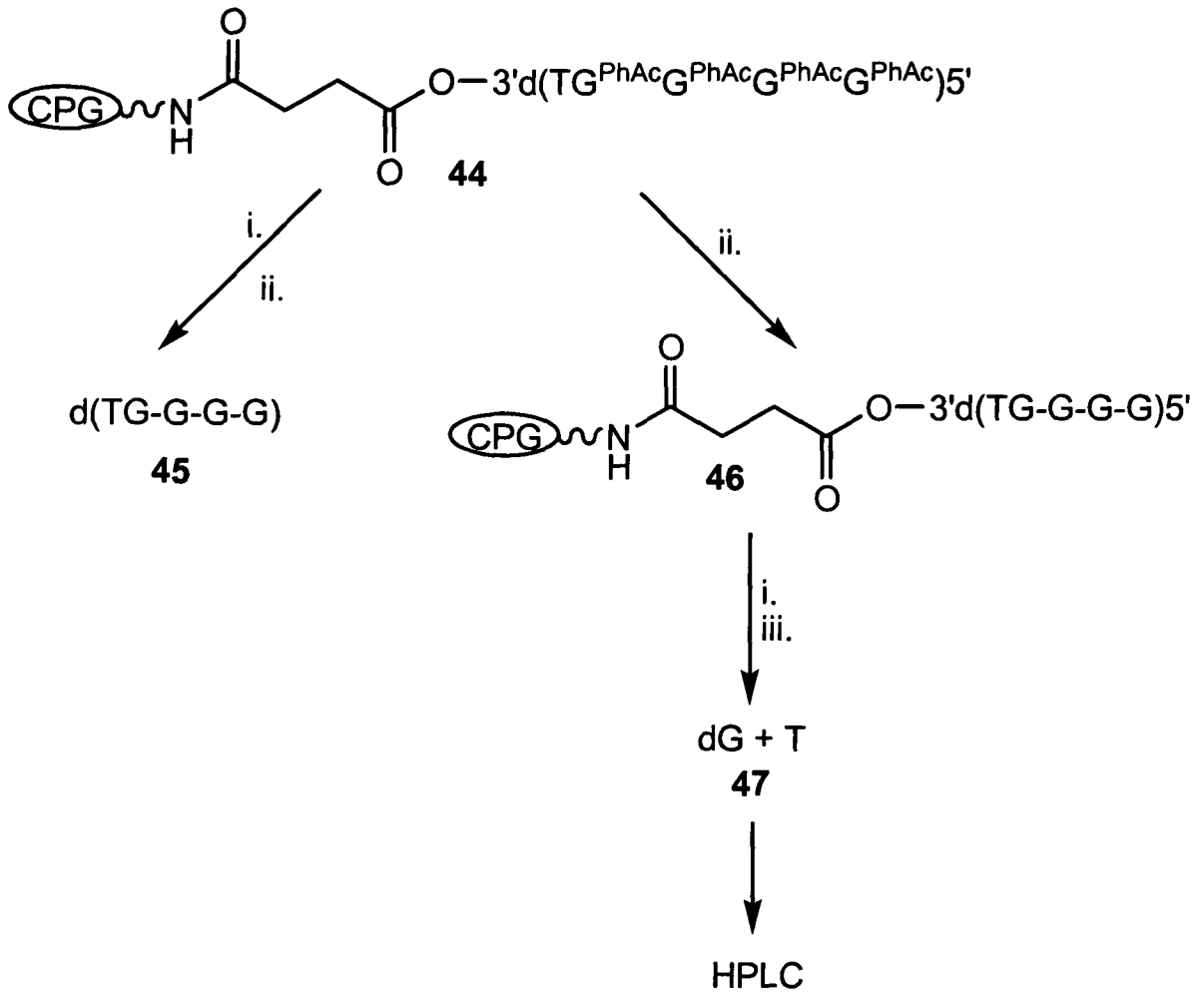
In the first step, the amino functionalized membrane was coupled with peptide spacer glutamic acid-(γ-*tert*-butylester)-(ε-aminohexanoic acid)-(ε-aminohexanoic acid) using Fmoc chemistry. Subsequent to coupling of the trypsin labile Glu-Lys handle, the membrane was mounted in an ASP 222 automated SPOT robot[39] and a grid of the desired format was spotted at each position. Further chain elongation was performed with Fmoc-protected PNA monomers to synthesize the desired PNA oligomers **43**. Finally, they were cleaved with bovine trypsin at the Glu-Lys handle and analysed by mass spectrometry.

1.2.3 Enzyme cleavable protecting groups in solid phase chemistry

1.2.3.1 Non-Swelling resins

In 1997, Waldmann and Reidel[40] reported the deprotection of phenylacetyl moieties from oligonucleotides immobilized on controlled pore glass beads using penicillin G acylase (Scheme 15). Pentanucleotide **44**, immobilized on controlled pore glass beads, was treated with the enzyme to give the deprotected pentanucleotide **46**. Subsequent treatment with ammonia released the pentanucleotide, which was analysed by digestion with phosphodiesterase and HPLC. Only deoxyguanidine (dG) and thymidine (T) **47** were found (*i.e.* no

phenylacetyl-protected nucleosides could be detected by HPLC), indicating that penicillin acylase removed the four phenylacetyl protecting groups quantitatively.



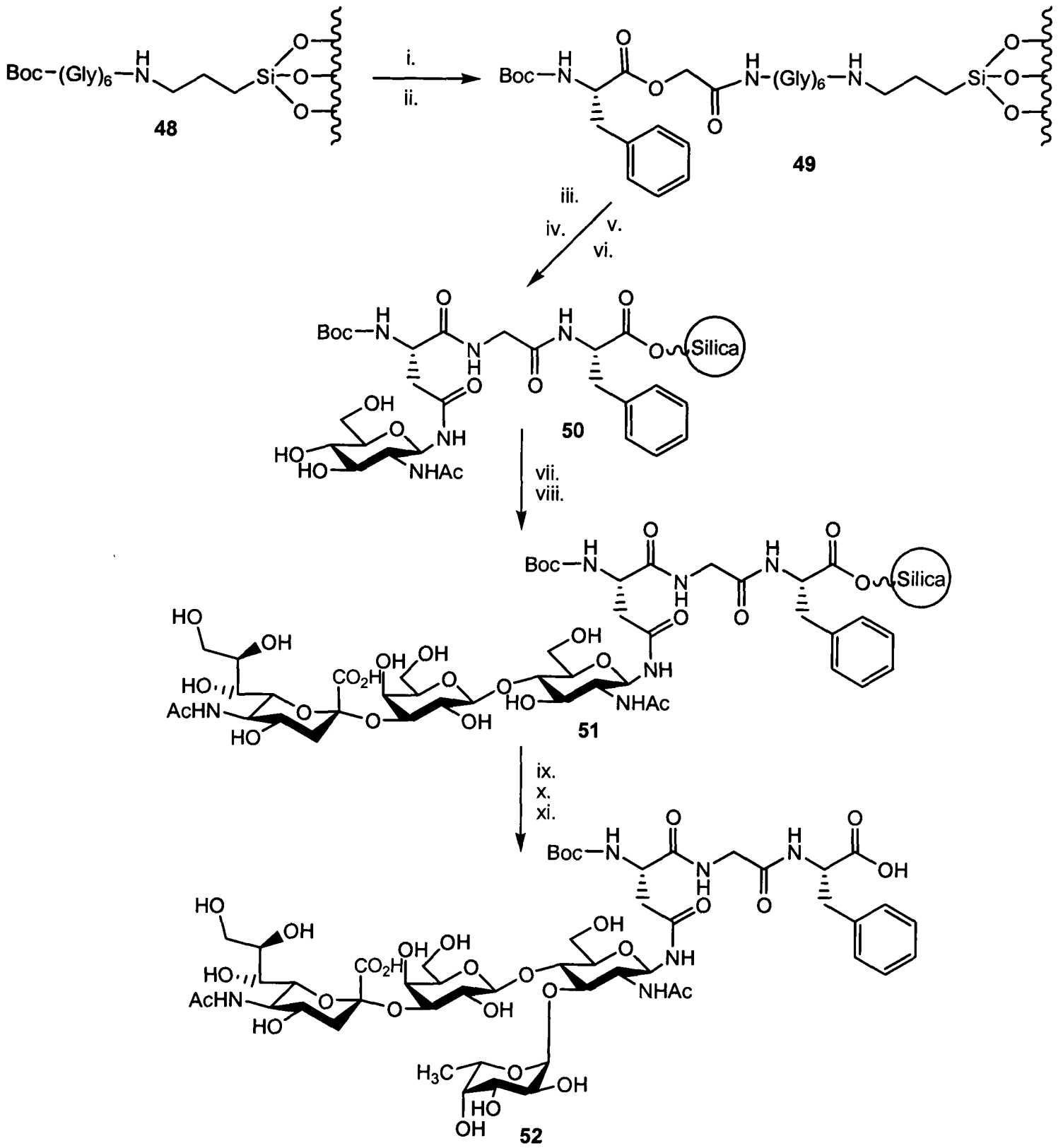
Scheme 15: *Reagents and conditions:* i. concentrated ammonia, 45 min, r.t.; ii. Penicillin G acylase pH 7, r.t., 2 h; iii. Phosphodiesterase from *Crotalus duriscus*, alkaline phosphatase from *E. coli*, TRIS buffer, $MgCl_2$, 37 °C, 12 h.

1.2.4 Enzyme cleavable linkers for release of polymer-bound substrates

1.2.4.1 Non-swelling resins

An example of an enzyme cleavable linker was reported by Wong *et. al.*[41] in 1994. They employed an α-chymotrypsin sensitive, phenylalanine ester linker for the preparation of a glycopeptide **52** using β-(1,4)-glycosyltransferase (50 kDa) and α-(2,3)-sialyltransferase which was released from solid support using α-chymotrypsin (22 kDa) (Scheme 16). Styrene- and sugar-based polymers were unsuitable, as they tend to swell which led to low coupling yields. Aminopropyl silica was chosen as the solid support, since it was compatible with aqueous and organic solvents and possessed a large surface area due to the non-swelling open

pore structure. In addition, amino propyl silica has a reasonable density of functional groups (~1.5 mmol g^{-1}).

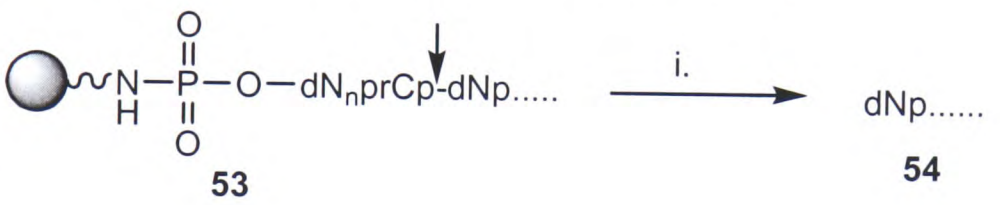


Scheme 16: *Reagents and conditions:* i. 25% TFA/CH_2Cl_2; ii. *O*-(*N*-*t*Boc-phenylalanyl)glycolic acid, BOP/HOBt, DIPEA; iii. 25% TFA/CH_2Cl_2; iv. Boc-Gly-OH, BOP/HOBt, DIPEA; v. TFA/CH_2Cl_2; vi. Boc-Asn(GlyNAcβ)-OH, BOP, DIPEA; vii. galactosyltransferase, UDP-Gal, HEPES pH 7.0, $MnCl_2$, 55%; viii.sialyltransferase, CMP-NeuAc, HEPES pH 7.0, $MnCl_2$, 65%; ix. α-chymotrypsin, H_2O pH 7.0; x. ultrafiltration; xi. fucosyltransferase, GDP-Fuc, HEPES pH 7.0.

The linker **49**, was constructed on a hexaglycine spacer **48** which was attached to the silica support to give a markedly reduced substitution of 0.2 mmol g^{-1} (*c.f.* ~1.5 mmol g^{-1}). The phenylalanine ester was coupled using the BOP/HOBt coupling procedure to give **49**. Subsequent, formation of the glycopeptide was achieved by

galactosyl transfer catalysed by galactosyltransferase in 55% yield, followed by sialylation to give the glycopeptide, **51** in good yield (65%). The desired product **52** was released from the solid support in high yield (95%) after fucosylation with fucosyltransferase.

Schmitz and Reetz[42] ultilised kieselguhr/polydimethylacrylamide resins for the synthesis of oligonucleotides on the solid phase using T4 RNA ligase. This rigid support consists of cross linked polydimethylacrylamide gel polymerized within the pores of macroporous kieselguhr (SiO_2) particles. The pores are freely permeated by large molecules and are suitable for enzymatic synthesis. RNase A selectively cleaved the last bound nucleotide at the ribose sugar **53** leaving a 3',5'-diphosphorylated oligomer behind on the resin (Scheme 17).



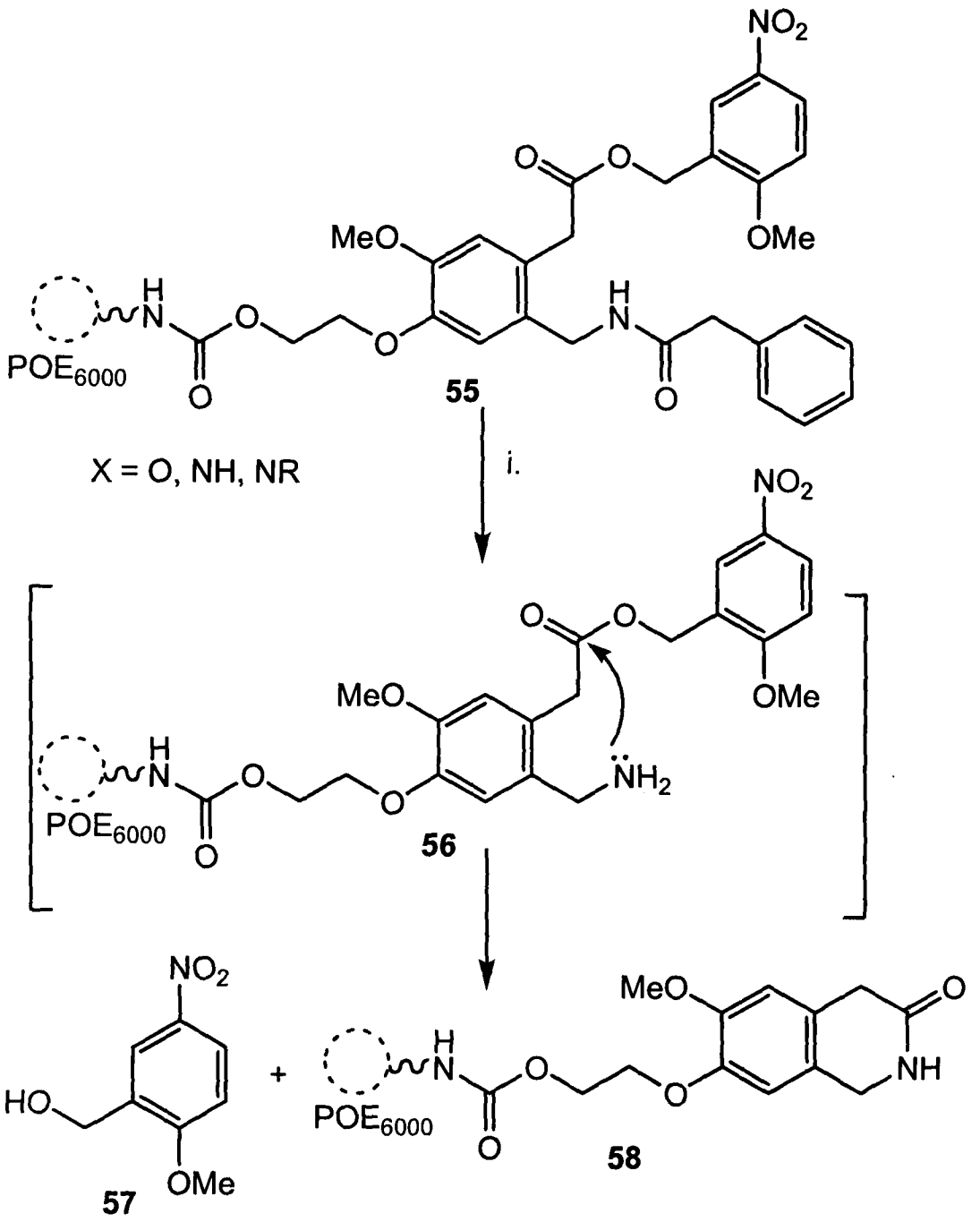
53 **54**

Scheme 17: *Reagents and conditions:* i. RNase A

1.2.4.2 Soluble polymers

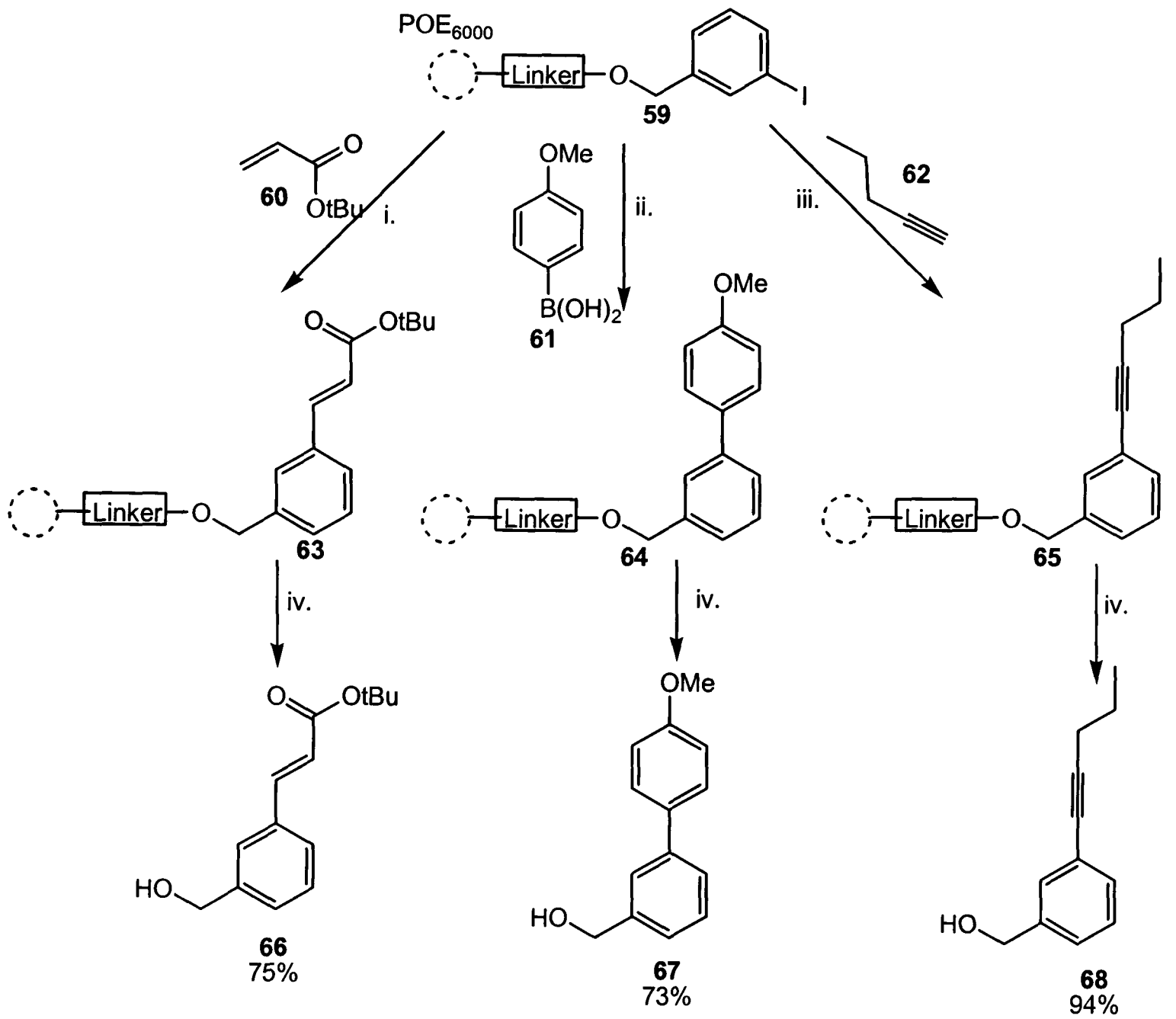
Waldmann *et. al.* have published two papers reporting the synthesis of a safety catch linker, **55** that can be cleaved by penicillin G acylase[43,44] (Scheme 18). Cleavage with penicillin G acylase gives the benzylamine intermediate, **56** which spontaneously cyclises to release the target molecule. Various solid supports (TentaGel, CPG (pore size 50 nm), $PEGA_{1500}$ and POE_{6000}), pH values, temperatures and co-solvents were investigated with a view to optimizing the enzyme cleavage yields. Although the solution phase analogue of **55**, was hydrolysed in excellent yield (90%); only low cleavage yields were obtained on TentaGel, PEGA and CPG resins (1, 13 and 10% respectively). It was thought that penicillin acylase (80 kDa) was too large to penetrate efficiently into the interior of the TentaGel beads.[28] The incorporation of longer spacers for CPG and PEGA resins were suggested, to improve enzyme cleavage. The use of soluble polyethyleneglycol (POE_{6000}), dramatically increased the cleavage yields of **57**, to 59%. POE_{6000} is soluble in many

organic solvents but can be precipitated in diethylether, filtered and washed to allow for easy separation of products.



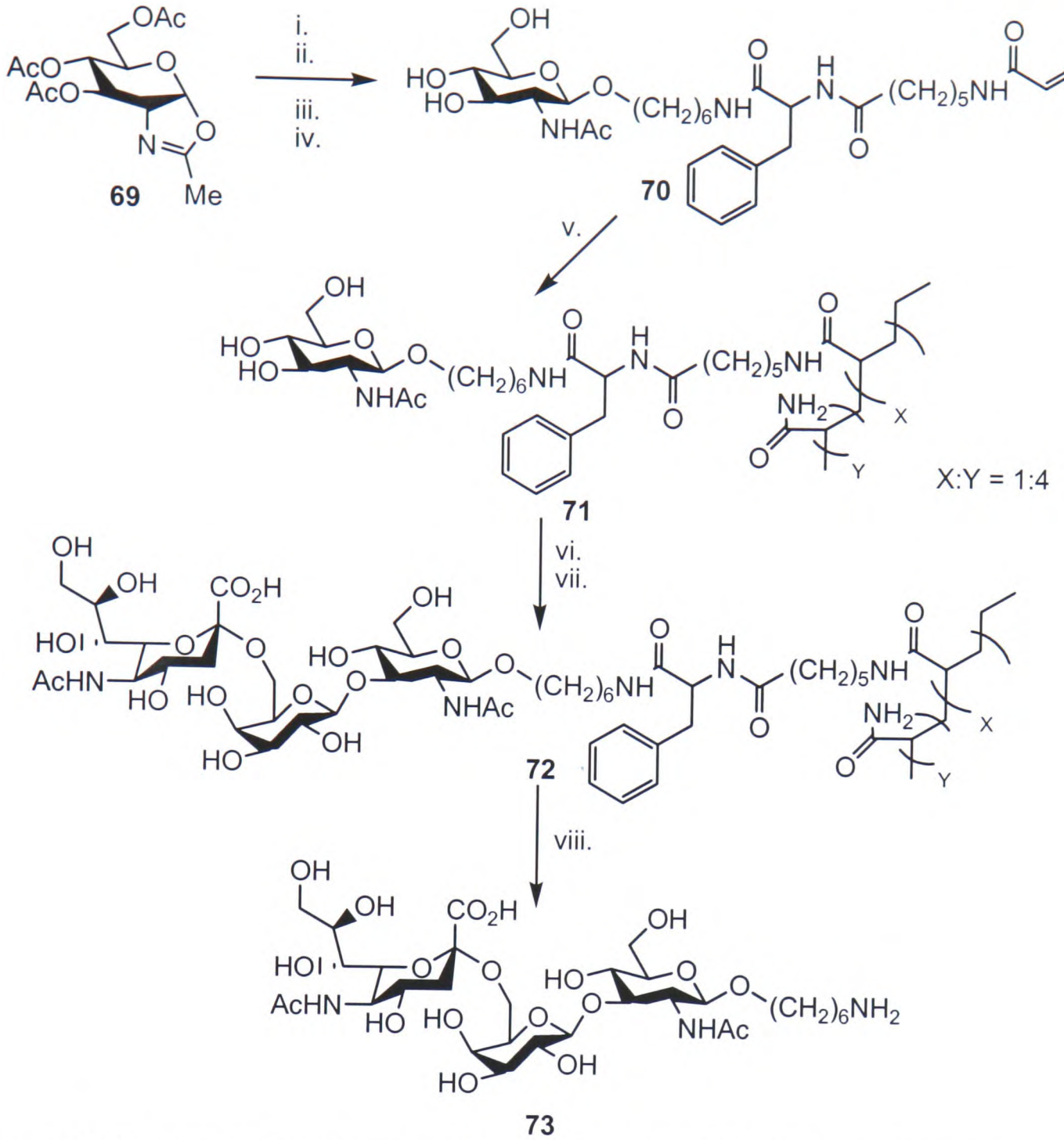
Scheme 18: *Reagents and conditions:* i. Penicillin G acylase suspension (pH 7.0), 10% methanol, 20 °C.

The application of the POE_{6000} linker was tested for a variety of transformations, including Pd(0) catalysed reactions. For example, Heck, Suzuki and Sonogashira reactions were carried out on polymer bound aryl iodide **59** to give cinnamic acid ester **63**, biphenyl **64** and alkyne **65** respectively (Scheme 19). Subsequent penicillin acylase hydrolysis was carried out to afford the respective products **66-68** in high yields and purity (>95%). Mitsunobu- and Diels-Alder reactions were also performed on this linker, with comparable success.



Scheme 19: *Reagents and conditions:* i. $[Pd(OAc)_2]$, Bu_4NBr, PPh_3, DMF/Et_3N/H_2O 9:1:1, 50 °C, 24 h, 91%; ii. $[Pd(PPh_3)_4]$, K_3PO_4, DMF, 80 °C, 20 h, 93%; iii. $[Pd(PPh_3)_2Cl_2]$, CuI, dioxane/Et_3N 2:1, 20 °C, 24 h, 97%; iv. Penicillin G acylase, pH 7.0, 10% methanol, 37 °C.

Yamada *et. al.* have published two papers[45,46] describing the enzymatic synthesis of oligosaccharide derivatives, **73**, which were cleaved from solid support with α-chymotrypsin (Scheme 20). Trisaccharide **72**, was generated on a water-soluble solid support, by enzymatic transformation with galactosyl- and sialyl-transferases. The oligosaccharide **73** was cleaved by α-chymotrypsin in good yield (overall 72% yield).



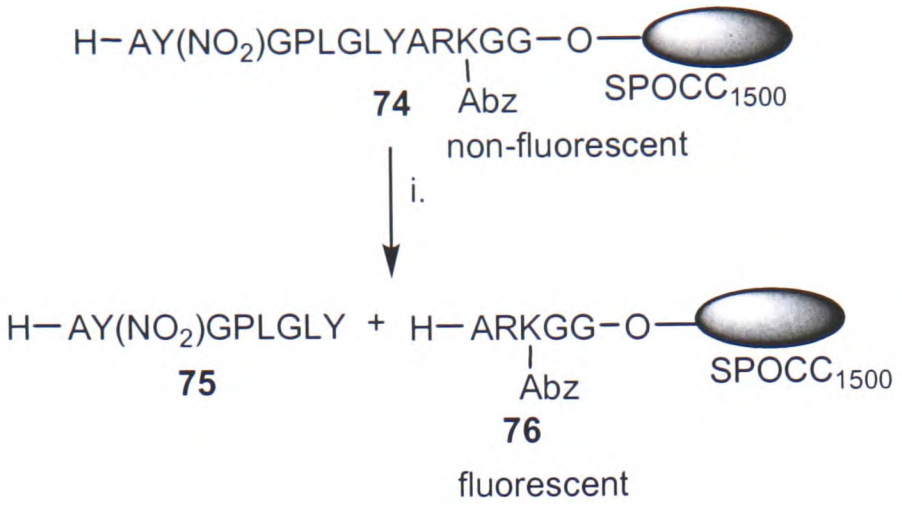
73

Scheme 20: *Reagents and conditions:* i. *Z*-Phe-NH-$(CH_2)_6$-OH, CSA, $(CHCl_2)_2$, 70 °C; ii. H_2, Pd/C, MeOH, 50 °C; iii. CH_2=CHCONH$(CH_2)_5$COOH, EtOH-benzene; iv. NaOMe (cat.), MeOH/THF; v. CH_2=$CHCONH_2$, TMEDA, APS, DMSO-H_2O, 50 °C; vi. Galactosyltransferase; vii. Sialyl transferase; viii. α-chymotrypsin, Tris-HCl buffer, pH 7.8, 48 °C.

1.2.4.3 Swelling Resins

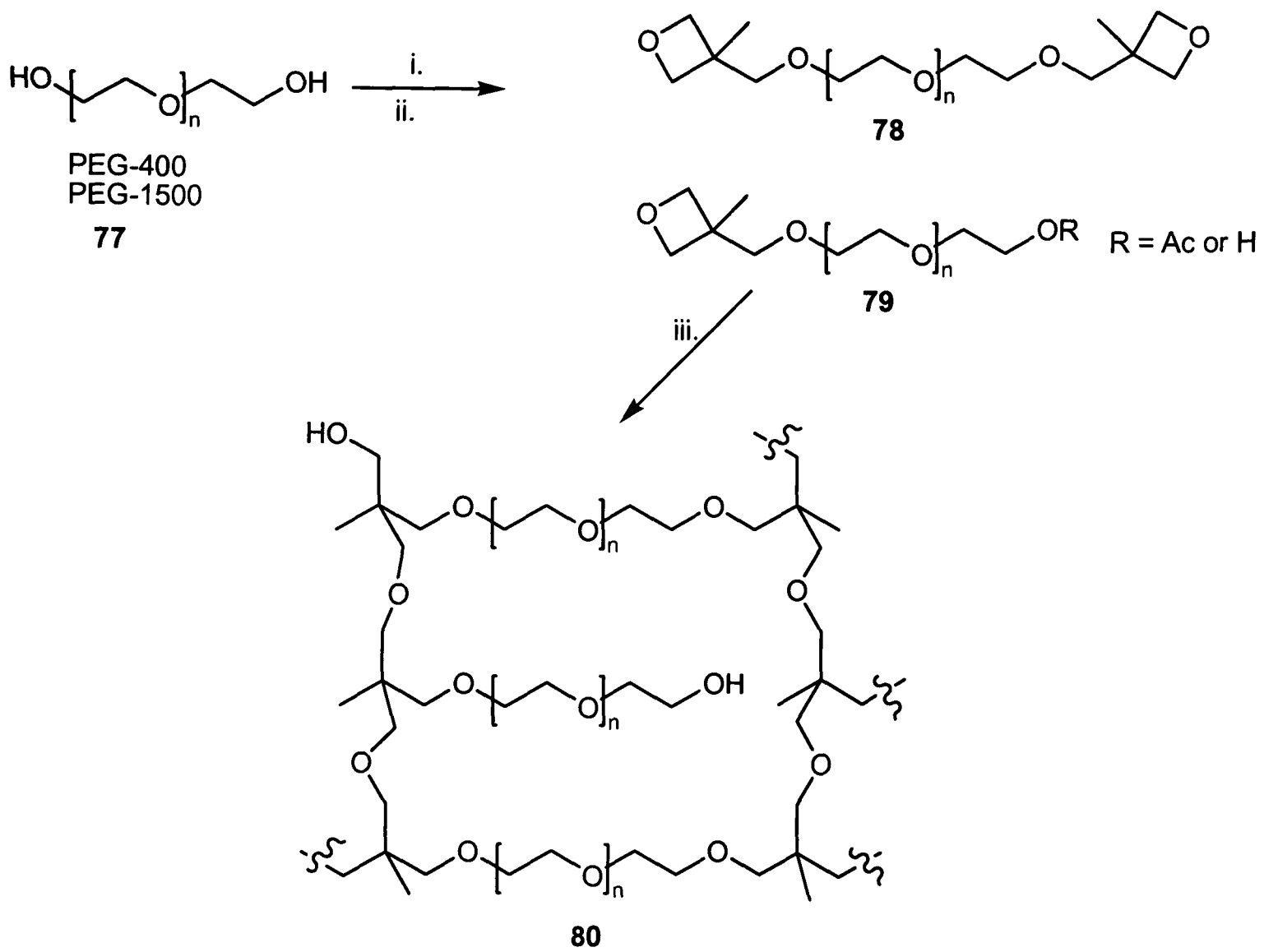
In 1999, Meldal *et. al.*[47] developed a novel poly(ethylene)glycol (PEG) based resin (SPOCC), for the enzymatic cleavage of a resin-bound decapeptide using a 27 kDa protease, subtilisin BNP'(Scheme 21). Edman degradation and HPLC of the residual fragment **76**, confirmed quantitative enzyme cleavage of **74** within 30 minutes. In contrast, the reaction of **74**, bearing a substrate for matrix metalloprotease MMP-9, a much larger protease of 72 kDa, was not cleaved by the enzyme. It was proposed that the enzymatic reaction was impeded by the resin

excluding the larger protease, indicating the importance of the permeability of the polymer in solid-phase enzyme reactions.



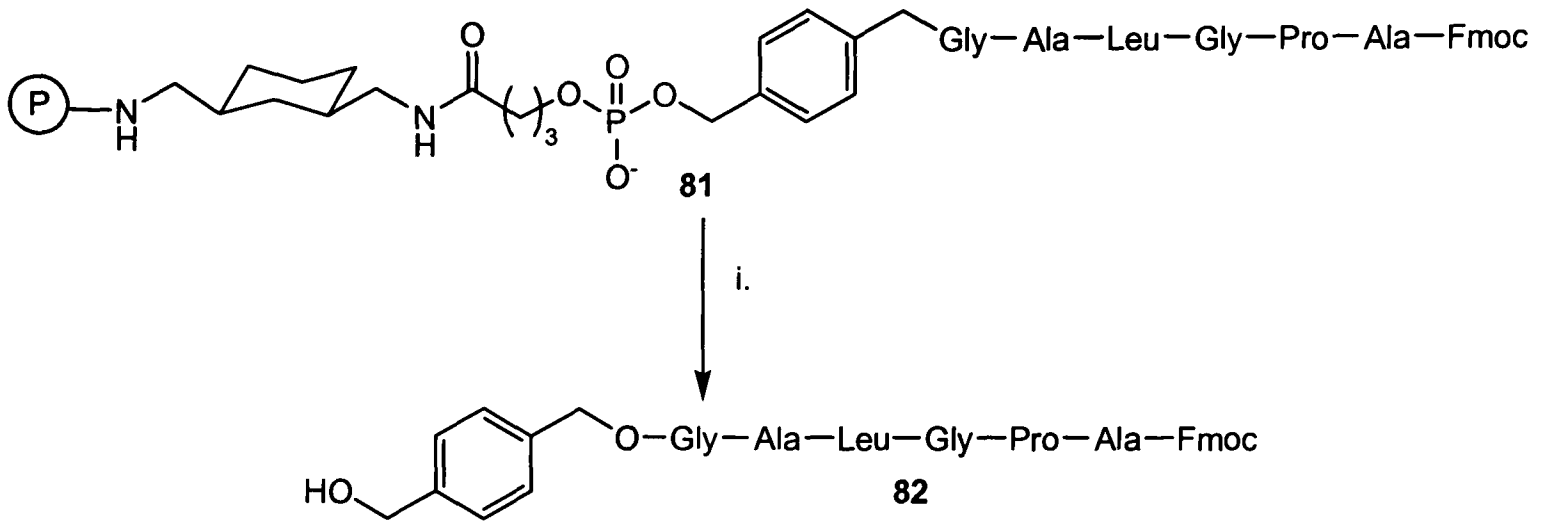
Scheme 21: *Reagents and conditions:* i. subtilisin BNP', phosphate buffer pH 7, 3 h.

SPOCC resin **80** is a polar support which is based on cross-linked poly(ethylene)glycol (PEG) terminally substituted with oxetane by cationic ring opening polymerization (Scheme 22). The polymer **80** was prepared using boron trifluoride diethyl etherate as initiator either *via* bulk polymerization in solution or suspension polymerization in silicon oil.



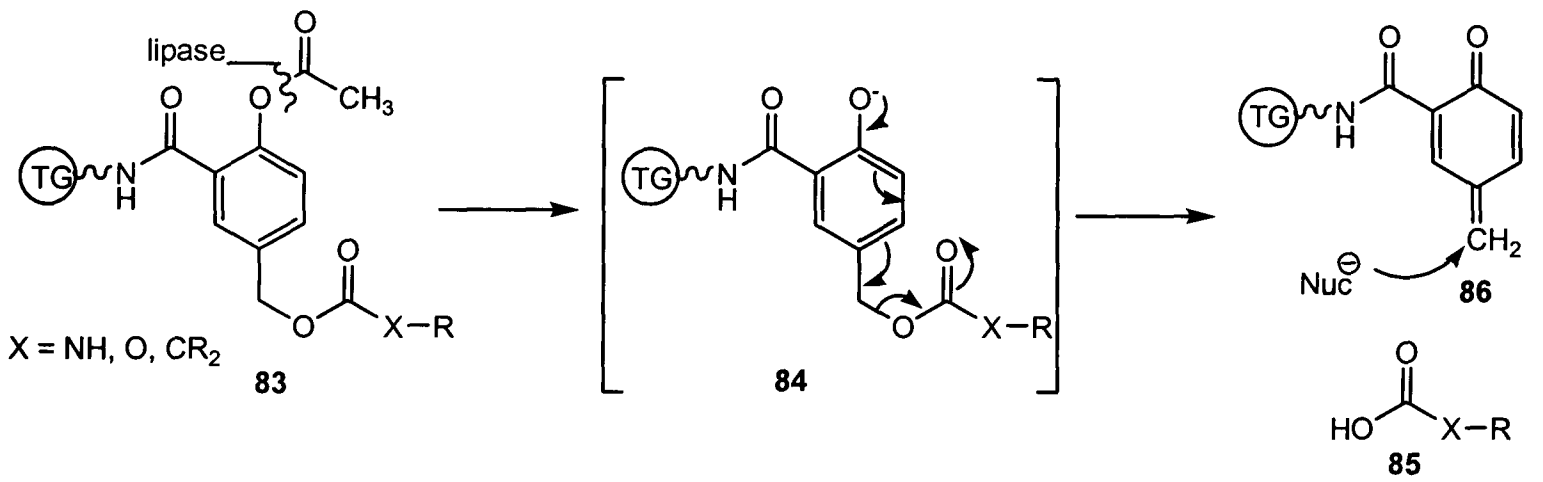
Scheme 22: *Reagents and conditions:* i. potassium hexamethyldisilazane, toluene/DMF (1:1), 15 min, r.t.; ii. (3-methyloxetan-3-yl)methyl 4-toluene sulfonate, 12 h, 75 °C; iii. boron trifluoride diethyl etherate.

The first esterase that was discovered to cleave linker groups on solid support was a phosphodiesterase. Elmore *et. al.*[48] reported the synthesis of a linker containing a phosphodiester moiety on a hydrophilic polyacrylamide resin (Pepsyn K resin) (Scheme 23). The utility of this linker **81** was demonstated by the generation of a collagenase substrate as the *p*-hydroxymethylbenzyl ester, **82** in good yield (83%), which was released from the solid support using calf spleen phosphodiesterase. This yield (83%) was only achieved after prolonged exposure to phosphodiesterase (7 days).



Scheme 23: *Reagents and conditions:* i. calf spleen phosphodiesterase, pH 5.7, 7 d, 83%.

Waldmann *et. al.*[49] have described the use of the 4-acyloxy-3-carboxybenzyloxy moiety as an esterase/lipase-labile linker for the synthesis of amines, alcohols and carboxylic acids (Scheme 24). Cleavage of the acyl group of **83** by lipase or esterase generates a phenolate **84** which fragments to give quinine methide **86** and the desired target compound **85**. TentaGel resin, a polystyrene resin with grafted polyethylene glycol units, was chosen as the solid support due to its compatibility with aqueous media.

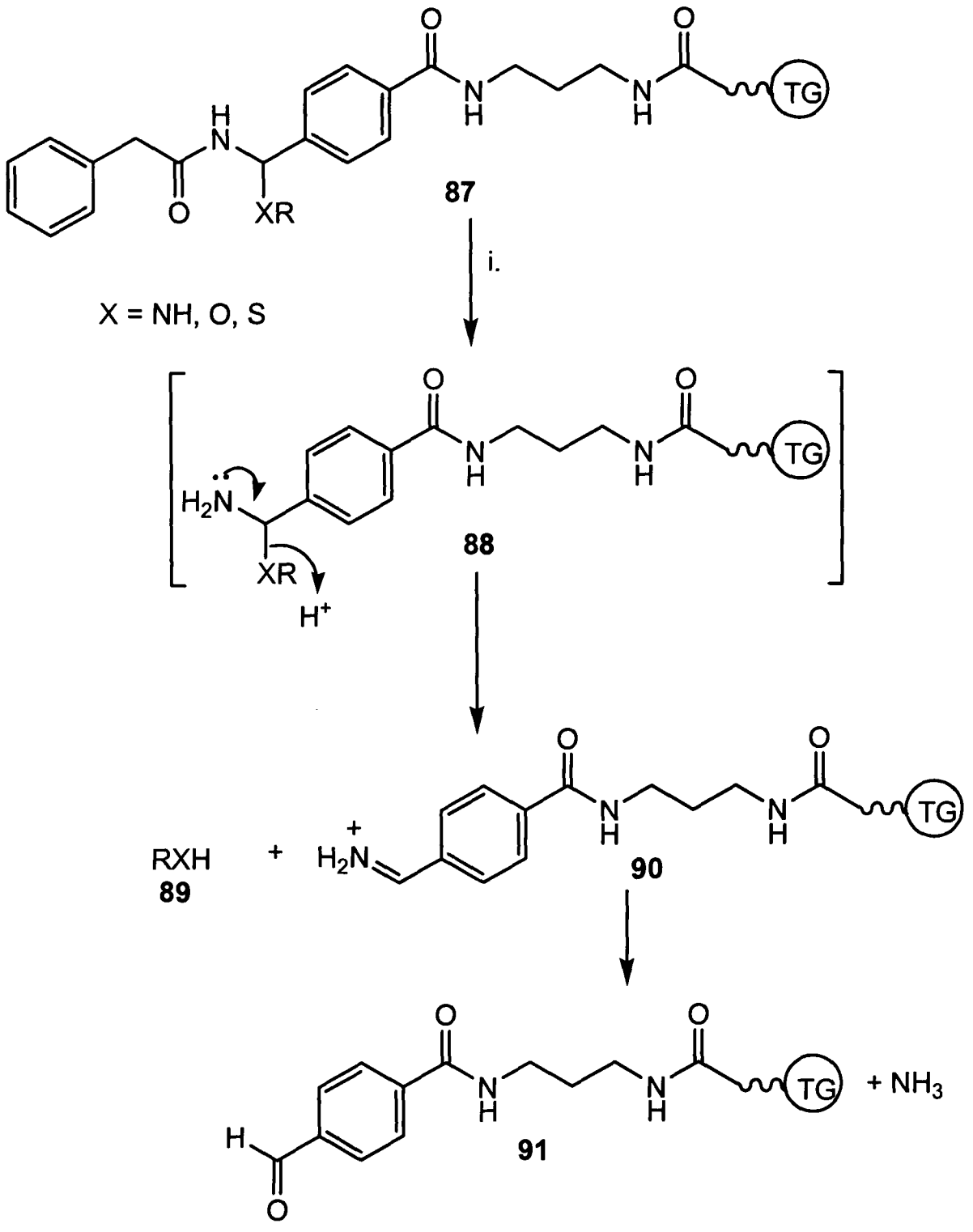


Scheme 24: *Mechanism of lipase catalysed cleavage of amines, alcohols and carboxylic acids from **83**.*

The best enzyme cleavage yields were obtained using recombinant lipase RB001-05. For example, leucine *tert*-butyl ester was cleaved in 73% yield, at pH 5.8 and 40 °C. Other examples include, carboxylic acids (*i.e.* tetrahydro-β-carbolines, 70-80%) and alcohols (68%). It was claimed that the substrate specificity of the enzyme guaranteed that only the intended ester was cleaved.

In 1996, Flitsch *et. al.*[50] reported the first synthesis of a penicillin G acylase cleavable linker **87** (Scheme 25). This linker was suitable for the immobilization of

alcohols and amines on solid support. Cleavage of the phenylacetamide moiety with penicillin G acylase affords the hemiaminal **88**, which spontaneously fragments in aqueous media to release the desired product **89**. The maximum enzyme cleavage yield obtained was 50% using TentaGel resin and RX = SEt. Alternatively, the linker **87** could also be cleaved using mild acid hydrolysis.



Scheme 25: *Reagents and conditions:* i. Penicillin amidase, phosphate buffer pH 7.5, 16 h, r.t.

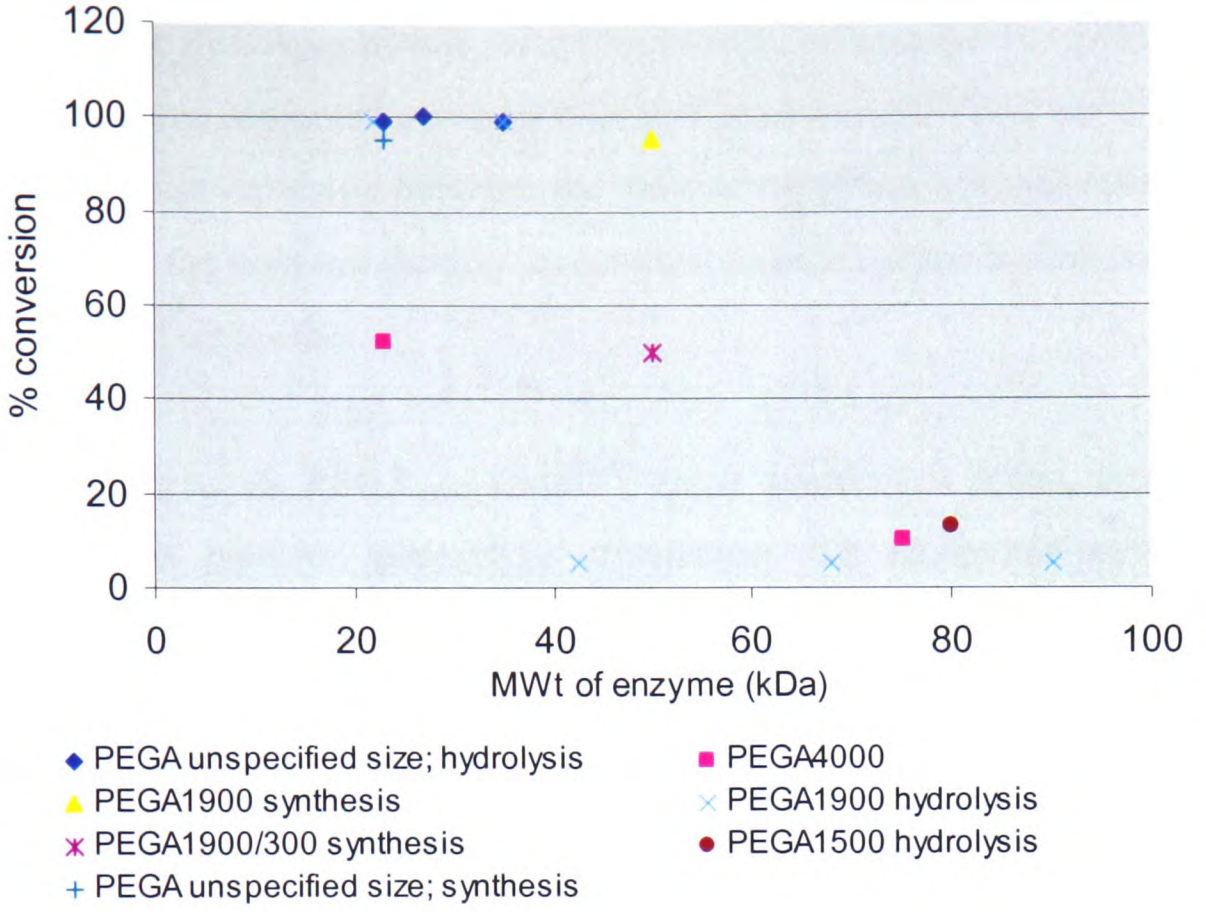
Concern over the permeability of resins to bio-molecules has led to various studies being carried out. Bradley *et. al.*[14] have carried out a systematic study into the permeability of enzymes to various solid supports [TentaGel resin, $PEGA_{1900}$, CPG (pore size,100 nm)]. Enzymes with a wide range of molecular weights (*e.g.*

MMP-12 (22 kDa), thermolysin (35 kDa), MMP-13 (42.5 kDa), clostridium collagenase (68 kDa), and NEP (90 kDa)) were incubated with the support bound 4-cyanobenzamide-Gly-Pro-Leu-Gly-Leu-Phe-Ala-Arg-OH. Confocal Raman microscopy was employed to monitor the absence/presence of the cyano stretching frequency. It was discovered that none of the enzymes could enter the TentaGel, $PEGA_{1900}$ was permeable to enzymes up to 35 kDa, while CPG was successful with enzymes up to 90 kDa. No mention was made of the substrate specificities of these proteases as presumably this would affect the amount of peptide cleaved by each enzyme.

1.2.5 Summary of biocatalysis in polymer supported synthesis/hydrolysis.

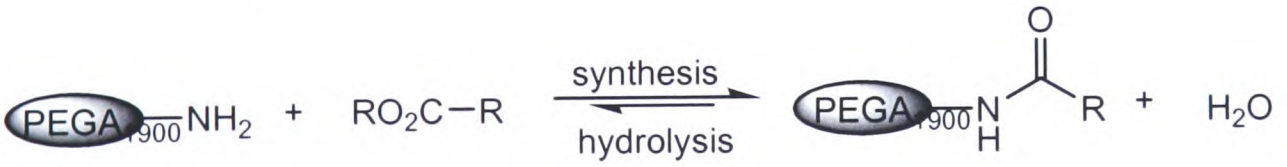
From the discrepancies in the literature reviewed above, it is obvious that the validity of solid phase mediated enzymatic transformations is still the subject of much debate (Appendix A). For example, it is generally agreed that only the surface sites of TentaGel are accessible to enzymes,[24,29,35,43,44] however two studies contradict this.[49,50] ArgoGel is also unsuitable for enzymatic transformations even with an enzyme of low molecular weight (23 kDa).[35] ArgoGel is very similar to TentaGel resin but possesses better swelling in aqueous media. However, it is thought that only surface sites are accessible to the enzyme.

The most commonly used solid support for enzymatic transformations is PEGA resin,[17,25-29,33,35,36,43,44] which is available in a variety of molecular weights. Graph 1 shows the relationship between percentage enzymatic conversion and the molecular weight of the enzyme for a variety of PEGA resins with different PEG lengths. It should be noted that where enzymatic conversion yields were not quoted, these points have been omitted from the graph.[25,27,28,30,33]



Graph 1: *Correlation between percentage enzymatic conversion and molecular weight of enzyme.*

In the case of $PEGA_{1900}$ resin for enzymatic hydrolysis (light blue cross), it can be seen that the resin is compatible with enzymes up to 35 kDa[14,34]. For synthesis on $PEGA_{1900}$ resin (yellow triangle), it seems that the resin is accessible to enzymes with a molecular weight of up to 50 kDa[17] (galactosyltransferase). This suggests that synthesis is favoured over hydrolysis, using solid support bound substrates. Recent reports by Flitsch *et. al.*[51,52] have shown that synthesis is favoured when reagents are bound to the solid support. This occurs because the synthesis/hydolysis equilibrium is shifted towards synthesis when reagents are immobilised on solid support (Scheme 26).



Scheme 26: *Reversible synthesis/hydrolysis on solid support.*

There are several explanations for the shift in equilibrium. Firstly, the presence of large excesses of starting materials shifts the equilibrium to synthesis according to Le Chatelier's principle. Secondly, $PEGA_{1900}$ resin is relatively hydrophobic

compared to the bulk aqueous media, thus hydrophobic starting materials are attracted into the $PEGA_{1900}$ matrix, which in turn favours synthesis. Lastly, the shift in equilibrium was explained by suppression of ionisation of the resin bound amine due to electrostatic repulsion between the amines which are held in close proximity to each other by the polymer matrix. In contrast, solution phase molecules can ionise independently of each other.

For hydrolysis on $PEGA_{4000}$ resin[31,36] (pink squares), it seems that there is a clear correlation between percentage conversion and molecular weight of the enzyme; as expected, percentage conversion is decreased with increasing molecular weight. It should also be noted that, increased crosslinking of the PEGA resin (purple star and yellow triangle) (*i.e.* $PEGA_{1900}$ and $PEGA_{1900/300}$) decreased the percentage enzymatic hydrolysis,[17] presumably caused by restricted accessibility of the enzyme to the resin due to smaller pore sizes. Finally, it can be seen that no correlation between PEGA size and percentage conversion exists, however, $PEGA_{1900}$ resin has produced the best enzymatic conversion yields.

Several resins have been specifically developed for use in enzymatic transformations on solid support, *e.g.* $EXPO_{3000}$[37] and SPOCC,[47] both of which are based on co-polymers of polyethylene glycol (PEG). However, only small enzymes were successful (27 kDa) and in the case of SPOCC resin, no cleavage was observed for an enzyme with a molecular weight of 72 kDa.

The use of macroporous resins for enzymatic transformations is limited but interest is slowly increasing in the use of these solvent independent resins.[14,23,26,28,40,42-44] In the case of Keiselguhr/polydimethylacrylamide resin, hydrolysis/synthesis has only been reported using fairly small enzymes (22-44 kDa).[26,28,42] Enzymatic hydrolysis on controlled pore glass (CPG) has been carried out with varying success (10% hydrolysis), however this was probably due to the small pore size used (50 nm).[43,44] A report by Bradley *et. al.*[14] supports this, with claims that enzymes up to 90 kDa are accessible to CPG with a pore size of 100 nm. Therefore, the pore size of CPG (100 nm) seems crucial for the accessibility of

enzymes. Other reports on the use of CPG[23,40] have not stated the pore size of the resin used. Aminopropyl silica has been used for enzymatic transformations using glycosyltransferases/ chymotrypsin (50 kDa/22 kDa) in moderate to good yields (55-65%).[41] However, no further studies have employed aminopropyl silica for enzymatic transformations.

Soluble polymers, such as polyacrylamide and polyethylene glycol have had limited but successful use in enzymatic transformations.[43-46] These polymers are soluble in aqueous media and selected organic solvents but can be precipitated in diethylether, filtered and washed so as to facilitate purification of products from the reaction mixture. So far, soluble polymers have not been widely used for organic synthesis and this is presumably due to the difficulties involved in precipitation after chemical elaboration.

1.3 Peptide aldehydes

Serine and cysteine proteases are potently inhibited by peptide aldehydes[53]. Inhibition arises from the formation of a hemiacetal or hemithioacetal tetrahedral adduct within the active site of the protease which mimics the proposed structure of the transition state (Scheme 3). Many serine and cysteine proteases have been implicated in a number of disease states; thus, their inhibition has been widely exploited medicinally.

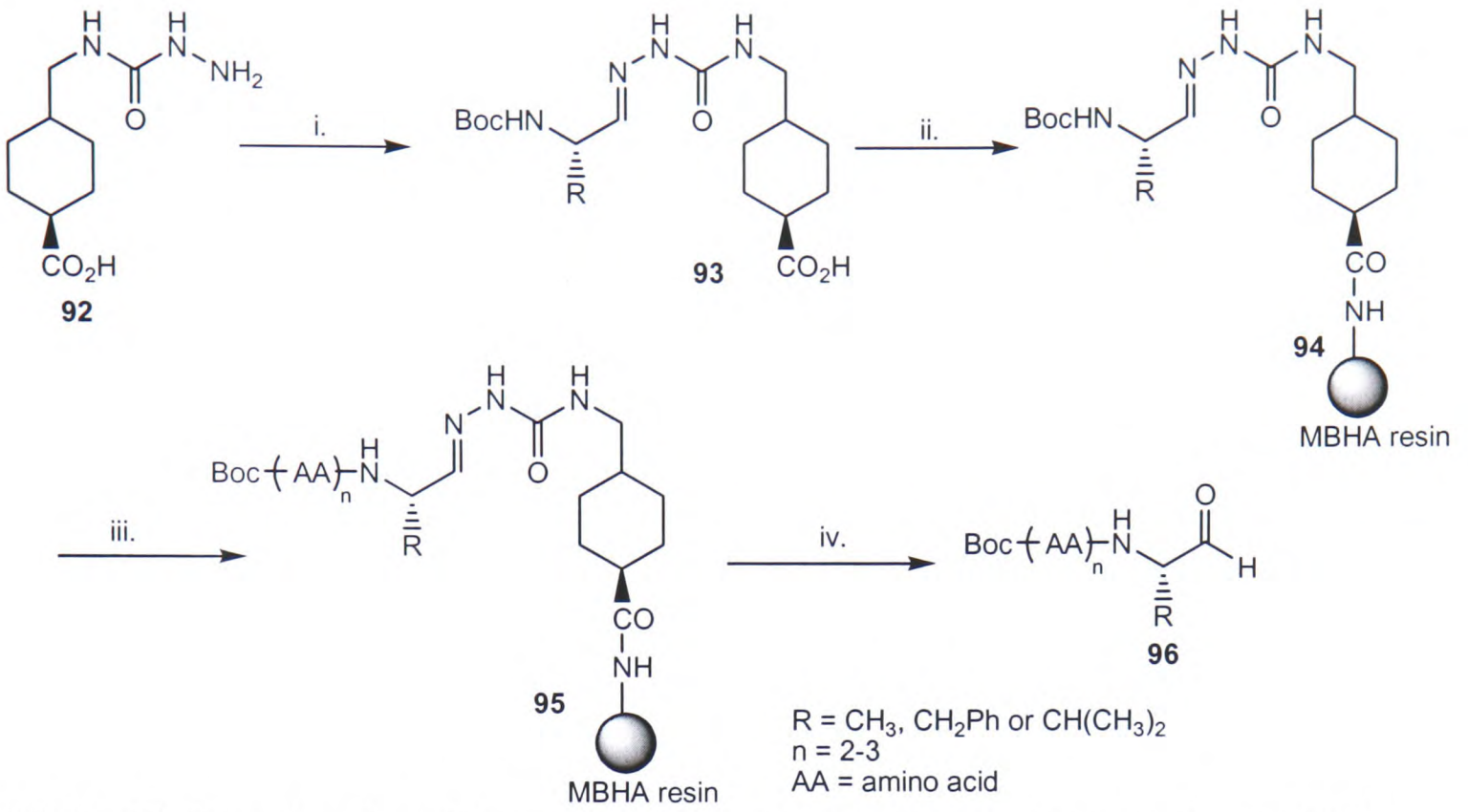
1.3.1 Methods for the preparation of C-terminal peptide aldehydes from solid support

Various methods for the solution synthesis of peptide aldehydes have been described[54-57]; however, relatively few methods have been developed for the solid phase synthesis of peptide aldehydes. The following criteria are necessary for a good peptide aldehyde linker:

- Must be cost effective.
- Must be easy to construct.
- Must be chemically stable under solid phase peptide synthesis conditions.
- Must give crude products with high purity, stereochemical integrity and high yield.
- Must be time economical.

Most of the methods available for the solid phase synthesis of peptide aldehydes are based on analogous solution phase methodologies. Aldehydes can be 'masked' or protected in a variety of ways; for example, as an alcohol, ester, amide, imine or ethylene, which may be converted to the aldehyde by oxidation or reduction, or they can be protected as *O,O*-acetals,[58,59] *O,N*-acetals,[60] *O,S*-acetals, *S,S*-acetals,[61] *Se,Se*-acetals[62] or as a thiazole.[63]

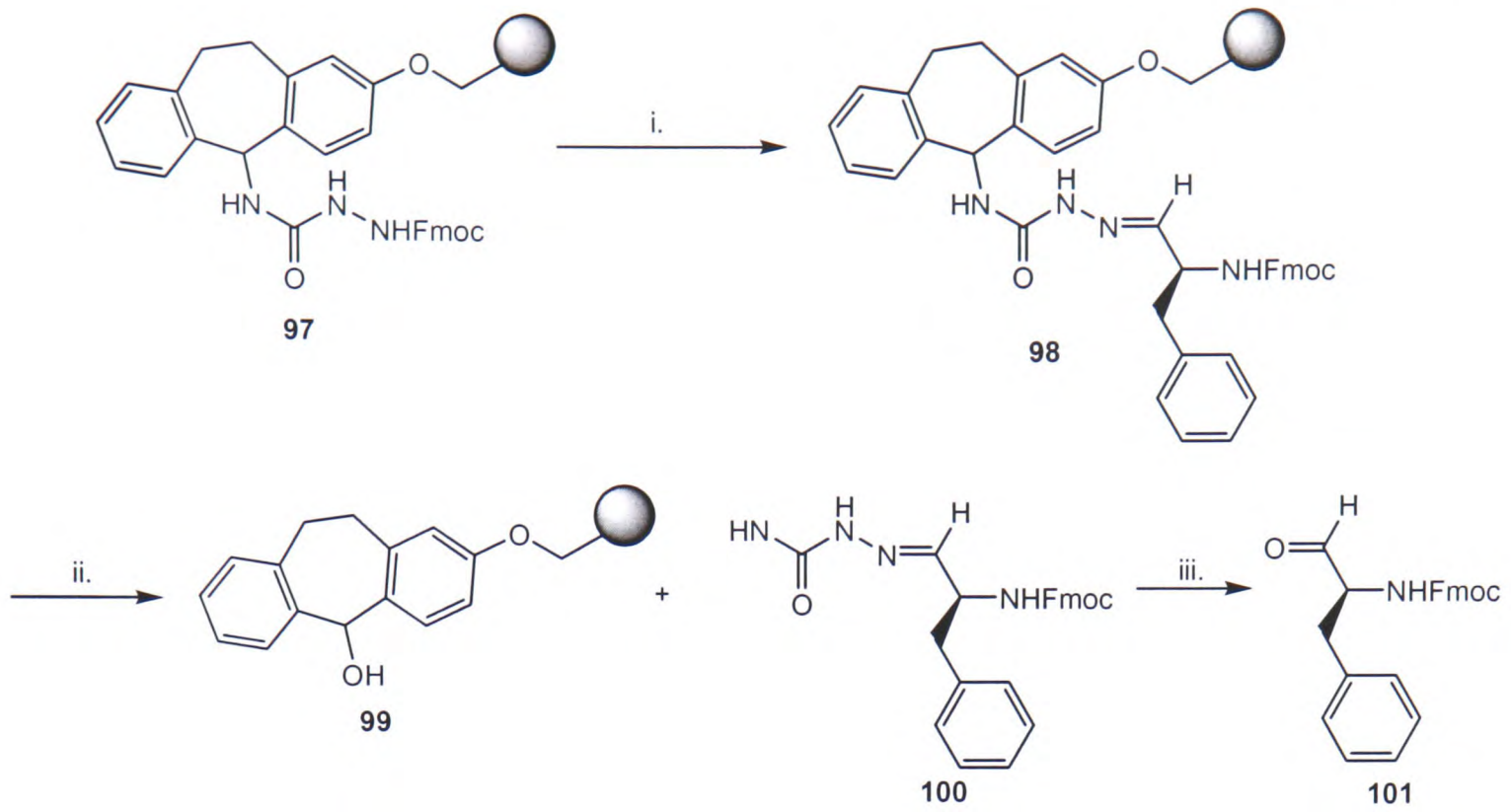
An early report by Webb *et. al.*[64] in 1992, described the use of a semicarbazone as a support bound protecting group for the synthesis of peptide aldehydes (Scheme 27).



Scheme 27: *Reagents and conditions:* i. Boc-amino aldehyde, NaOAc, ethanol, reflux; ii. MBHA resin, BOP; iii. peptide elongation; iv. dilute aqueous acid, formaldehyde.

The appropriate Boc-protected amino aldehyde was protected as its semicarbazone **93** by treatment with **92**. **93** was coupled to acid labile 4-methylbenzhydrylamine (MBHA) resin using benzotriazol-1-yloxytrisdimethylamino-phosphonium hexafluorophosphate (BOP) reagent to give **94**. After peptide synthesis, **95** was treated with aqueous acid/formaldehyde to generate the peptide aldehyde **96** from the support. Several examples of peptide aldehydes were prepared using this procedure; however no reference to the optical purity of the resulting peptide aldehydes was made.

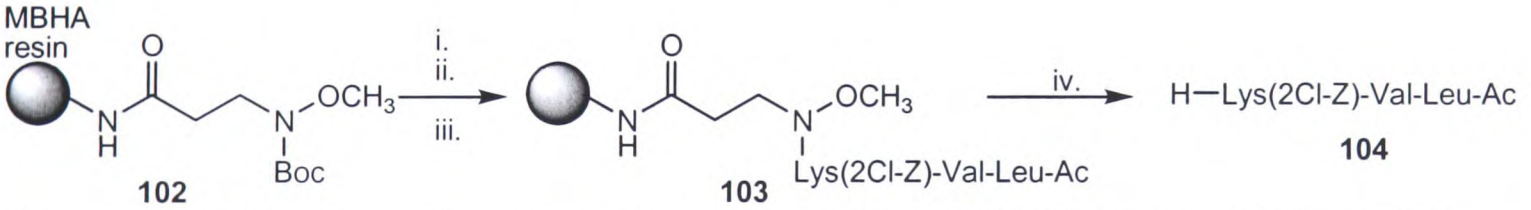
In a later publication, Patterson and Ramage[65] investigated linker **97** based on a dibenzosuberyl system for the synthesis of peptide semicarbazones **100** which can be converted into peptide aldehydes **101** (Scheme 28)



Scheme 28: *Reagents and conditions:* i. Fmoc-phenylalanine, DIPEA, 90%, 5 h; ii. TFA/H_2O (9:1), 1.5 h; iii. pyruvic acid.

This methodology was effective in generating peptide semicarbazones **100** and aldehydes **101** with no epimerisation at the *C*-terminal chiral centre. Despite this, the method has not been widely applied.

A more useful method for the solid phase synthesis of peptide aldehydes was inspired by the use of Weinreb amides for the solution phase synthesis of amino aldehydes[66]. Martinez *et. al.*[67,68] have prepared one such linker **102** and synthesized tripeptide aldehydes, such as **104**, on a MBHA resin (Scheme 29).

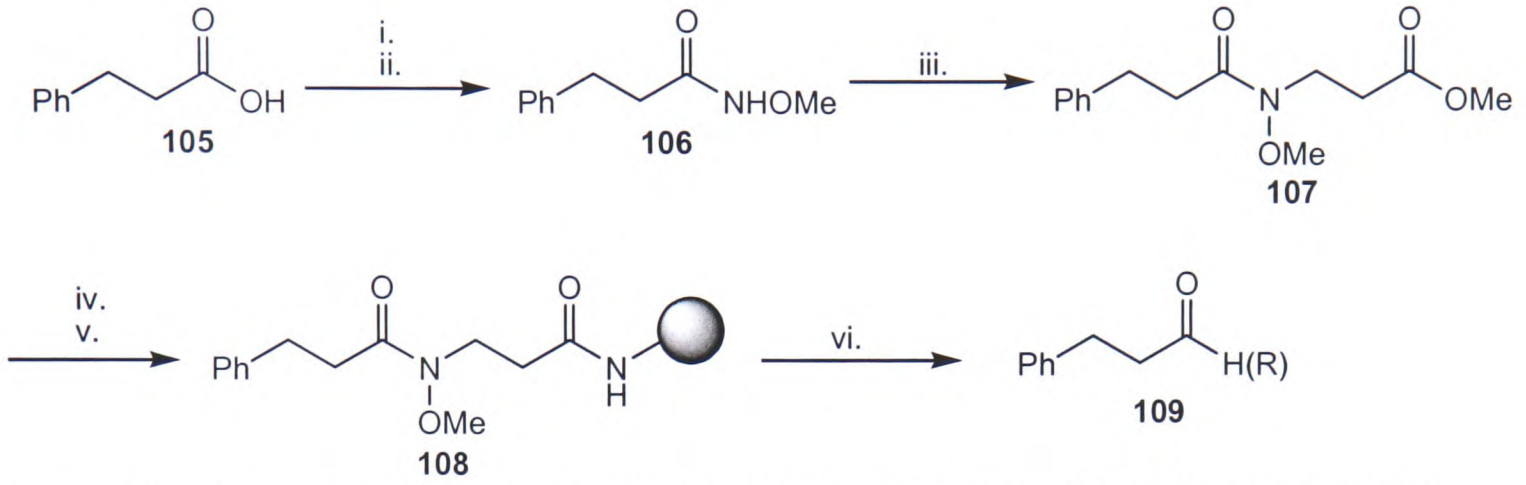


Scheme 29: *Reagents and conditions:* i. Boc synthesis, BOP/DIPEA, Boc-AA-OH; ii. Boc deprotection; iii. acetylation; iv. $LiAlH_4$, THF, 0 °C.

The tripeptide aldehyde **104** was removed from the solid support by reduction with lithium aluminium hydride. The crude peptide aldehyde **104** was studied by reverse-phase HPLC and ^{1}H NMR. Examination of the ^{1}H NMR spectrum revealed the presence of a single aldehyde proton signal, indicating the absence of epimerisation. Purification of the peptide aldehyde **104** was carried out by flash

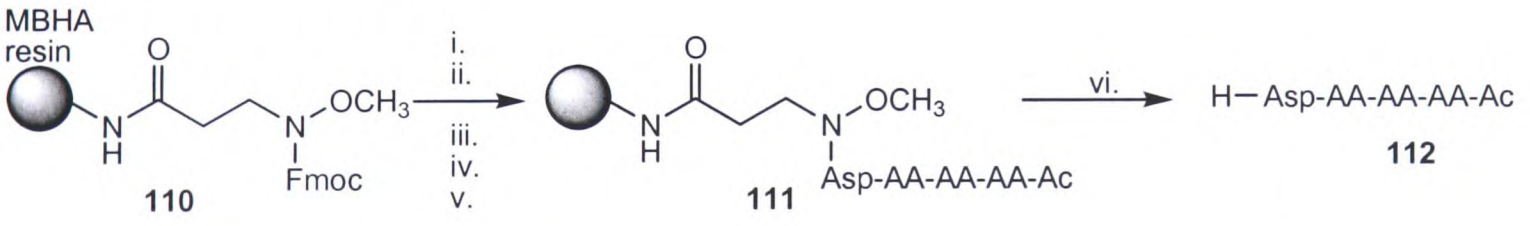
chromatography on silica gel (0.1% pyridine as eluent) or by reverse phase HPLC. Both purification methods resulted in a loss of optical purity as confirmed by appearance of two peaks in the aldehyde proton region of the ^{1}H NMR spectra.

At around the same time, Armstrong and Dinh[69] were also developing a Weinreb type linker for the synthesis of simple aldehydes and ketones **109** (Scheme 30).



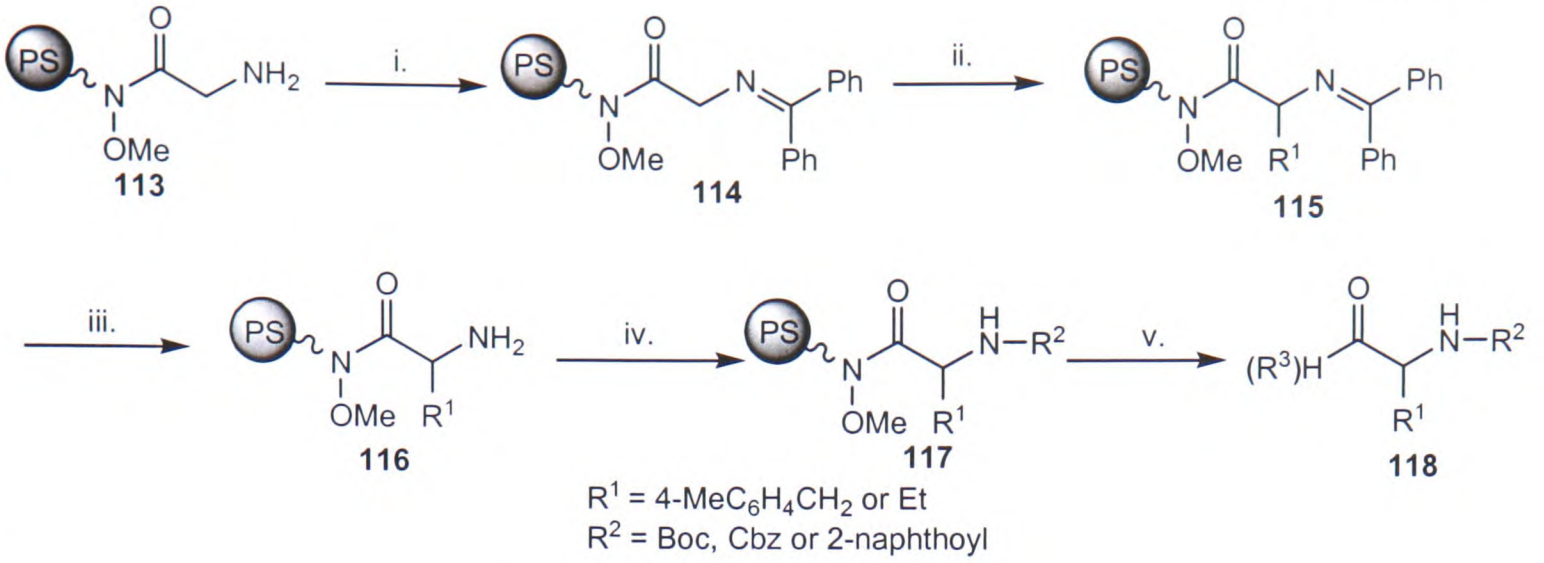
Scheme 30: *Reagents and conditions:* i. pivaloyl chloride, NEt_3, THF, 0 °C, 2.5 h; ii. $MeONH_3Cl$, NEt_3, 0 °C-r.t., 1 d; iii. cat. *t*BuOK, methylacrylate, PhMe, 70 °C, 8 h (83%); iv. LiOH, THF, r.t., 1 h (95%); v. Polymer-NH_2, DCC, HOBt, DMF, r.t., 1 d, (>95%); vi. $LiAlH_4$, THF, 5 °C, 15 h or RMgX, THF, 15 h, r.t.-60 °C.

Tong and Hong[70] have employed a Weinreb amide linker **110** for the synthesis of tetrapeptide aspartyl aldehydes **112** (Scheme 31). Reverse phase HPLC analysis of the crude peptide aldehyde **112** revealed some impurities; however, no further purification was carried out. No reference was made to the optical purity of the crude peptide aldehydes **112**.



Scheme 31: *Reagents and conditions:* i. 20% piperidine/DMF; ii. Fmoc-Asp(O*t*Bu)-OH, HATU, DIPEA; iii. Fmoc synthesis, PyBOP, HOBt, DIPEA; iv. acetylation; v. TFA, scavenger mixture; vi. $LiAlH_4$, THF, -20 °C.

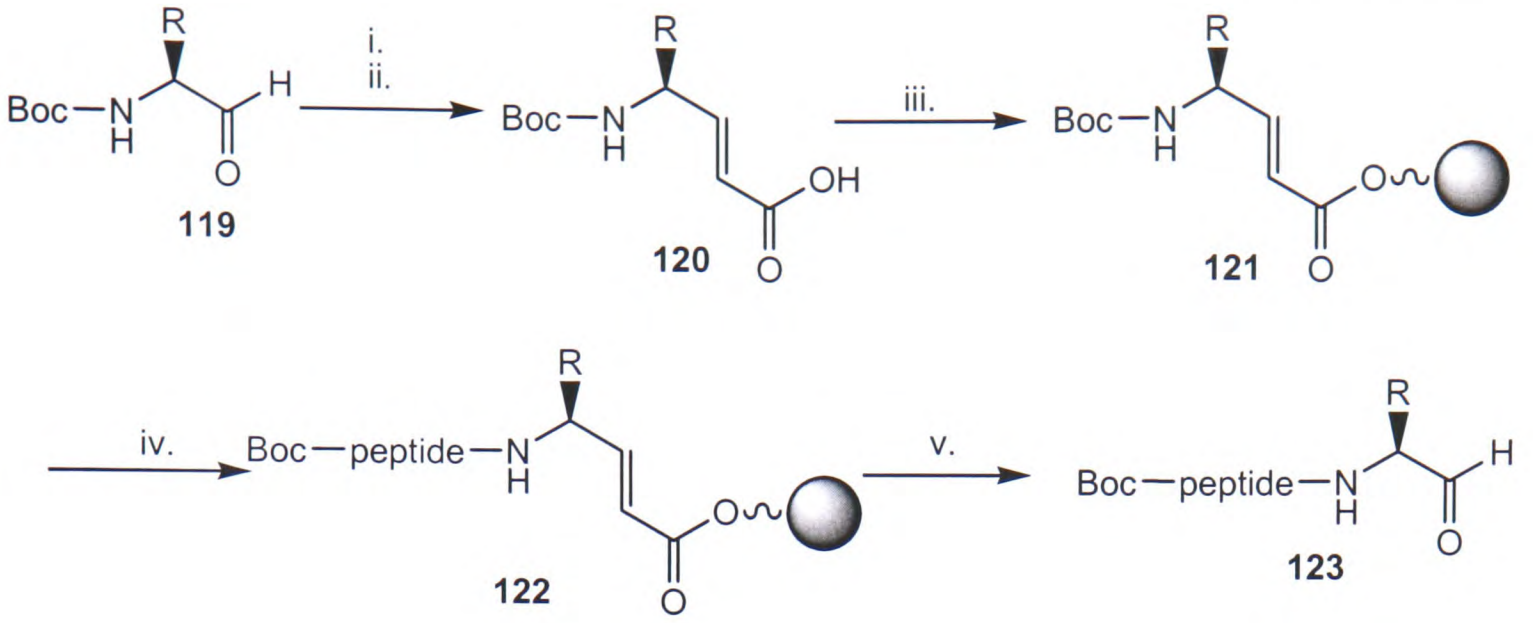
In 2000, O'Donnell *et. al.*[71] reported the solid phase synthesis of unnatural amino aldehydes, amino ketones, peptide aldehydes and peptide ketones **118** (Scheme 32).



Scheme 32: *Reagents and conditions:* i. benzophenone imine, 1-methyl-2-pyrrolidinone, 24 h, r.t.; ii. 1,4-methyl benzyl bromide or ethyl iodide, methyl-2-pyrrolidinone, dichloromethane, BTPP, 24 h; iii. 1 M HCl, THF, 4 h; iv. amine protection; v. R^3MgX or DIBALH, THF, 2 h then 1M HCl:THF (1:1), 15 min.

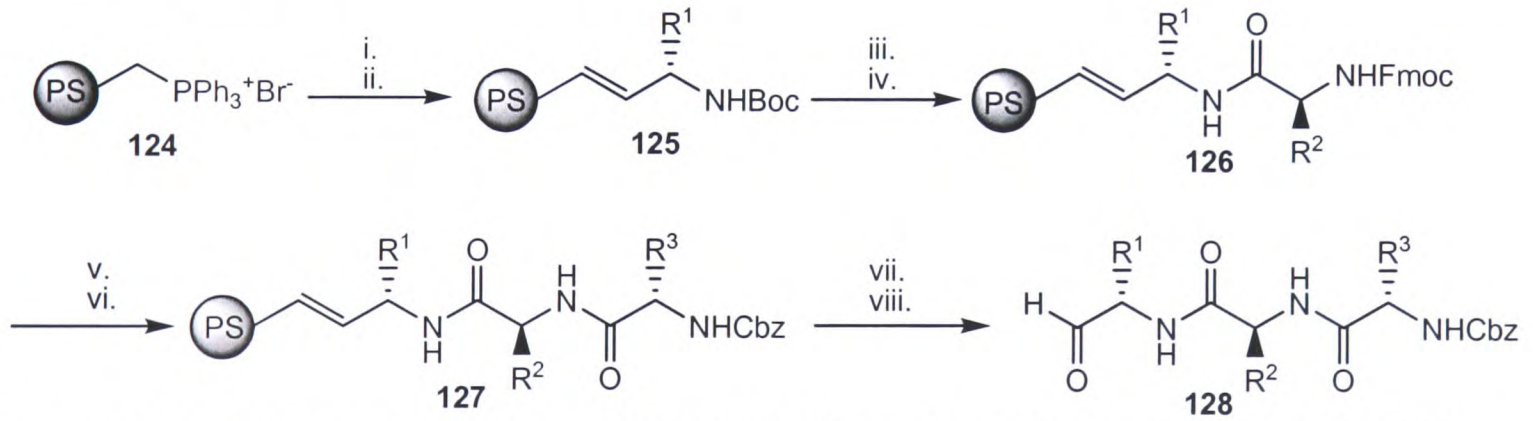
The synthesis of compound **118** was achieved by the addition of an unnatural side chain to a benzophenone imine-activated Weinreb resin bound glycine **114** to generate **115**. Subsequent hydrolysis of the alkylated imine **115**, followed by protection of the resulting amine **116** and cleavage with either DIBAL-H or Grignard reagent resulted in aldehyde or ketone **118** respectively (55-87% yield). The degree of epimerisation at the α-stereocentre was dependent on the conditions used for alkylation of **114** (*i.e.* choice of base and alkyl halide). It is now possible to obtain Weinreb amide linker from various commercial sources,[72] which is a testament to the success of this method.

The use of ozonolysis to generate peptide aldehydes from solid support was first exploited by Martinez et. al.[73] (Scheme 33). The protected α,β-unsaturated γ-amino acid **120** was prepared using a Wittig reaction between carboethoxymethylene triphenylphosphorane and protected amino aldehyde **119**. A model tripeptide aldehyde Boc-Phe-Val-Ala-H was prepared in high purity with no detectable trace of racemization (within the limits of ^1H NMR).



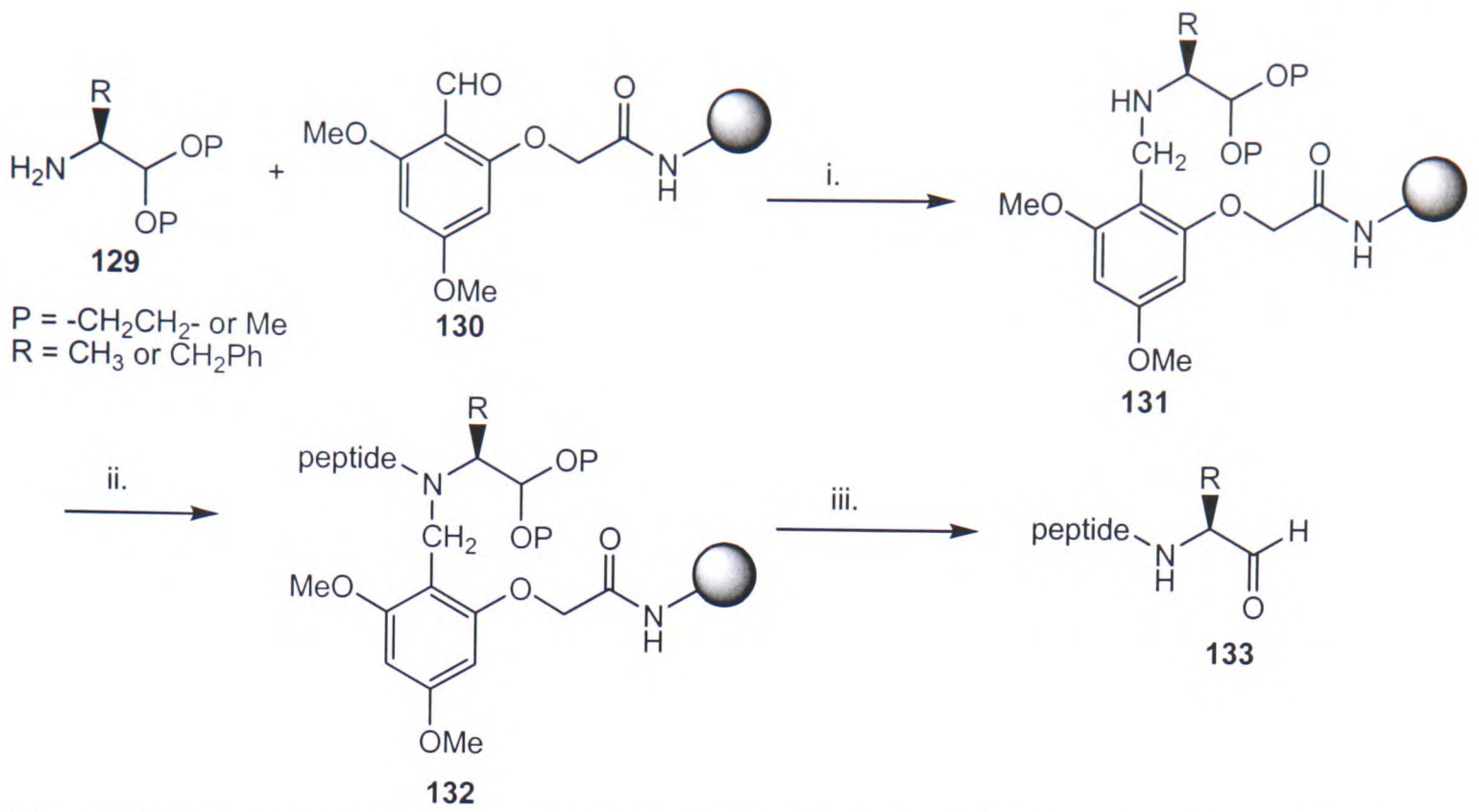
Scheme 33: *Reagents and conditions:* i. carboethoxymethylene triphenylphosphorane, toluene, 80 °C, 1 h; ii. NaOH, EtOH, 5 h; iii. MBHA resin, BOP; iv. peptide elongation using BOP reagent; v. O_3, DCM, -80 °C, 20 min then thiourea, methanol, 10 min.

This method of generating aldehydes is limited to sequences which are stable to ozonolysis. Nevertheless, Hall and Sutherland[74] have demonstrated the utility of ethylenic linkers by preparing 27 tripeptide aldehydes **128** (Scheme 34). The peptide aldehydes **128** were obtained as single diastereomers with purity >90% (by LCMS). The limitations of this method were illustrated when methionine was incorporated into the tripeptide. Two products were observed after ozonolysis; the corresponding sulfoxide and sulfone in a 1:1 ratio. This indicated that the side chain sulphide was oxidised by the ozone.



Scheme 34: *Reagents and conditions:* i. NaHMDS, THF, r.t., 15 min; ii. Boc-amino aldehyde, THF, r.t., 20 h; iii. 25% TFA/DCM, r.t., 20 min; iv. Fmoc-AA-OH, PyBOP, DIPEA, DMF, r.t., 2 h; v. 20% piperidine, DMF, r.t., 15 min; vi. Cbz-amino acid, PyBOP, DIPEA, DMF, r.t., 2 h; vii. O_3, DCM, -78 °C, 5 min; viii. Me_2S, DCM, 3 h.

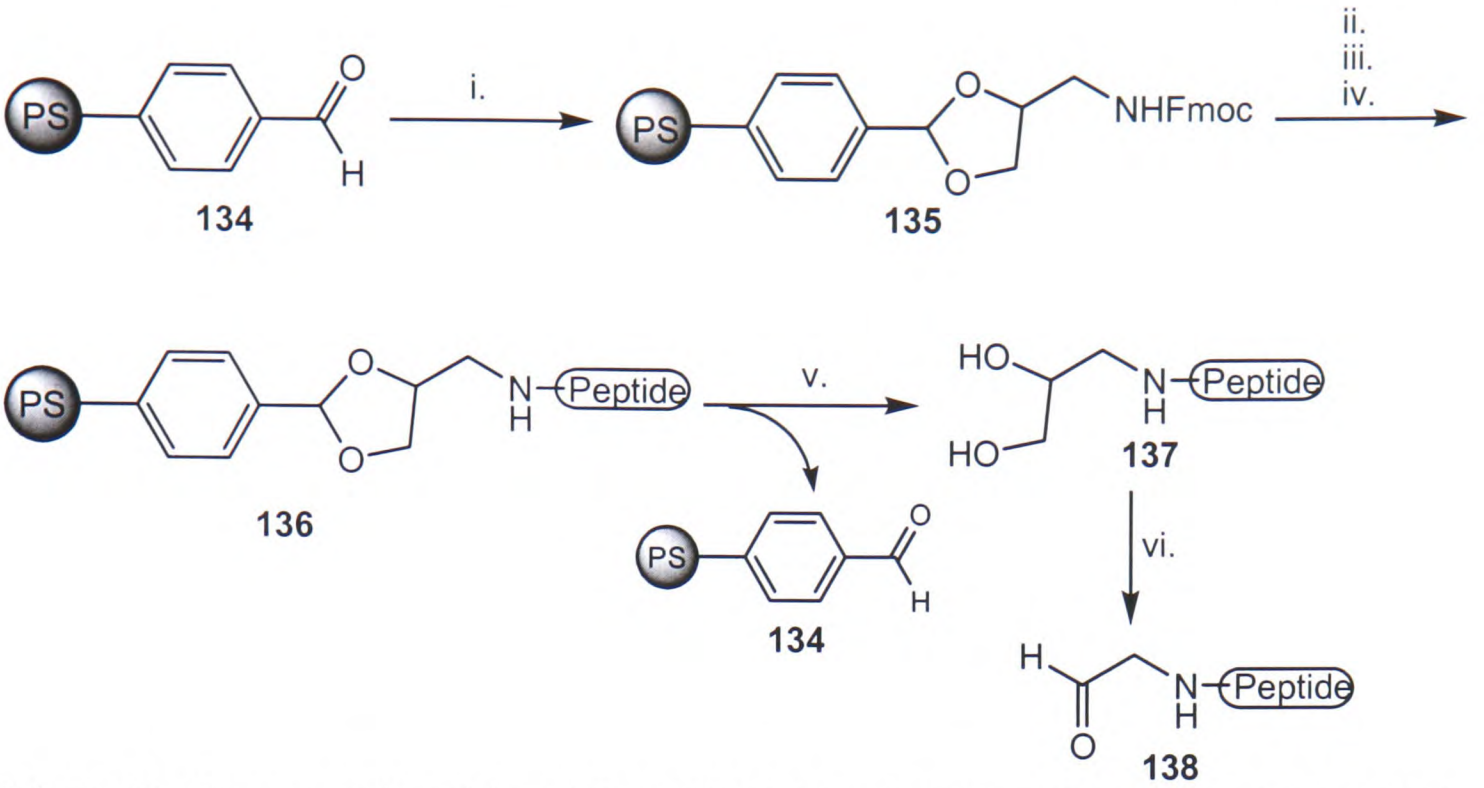
Barany *et. al.*[75,76] have reported the synthesis of peptide aldehydes **133** from amino acetals using a backbone amide linker (BAL) **131** (Scheme 35).



Scheme 35: *Reagents and conditions:* i. $NaBH_3CN$, DMF/HOAc, 25 °C, 1-12 h; ii. chain elongation; iii. TFA/H_2O (19:1).

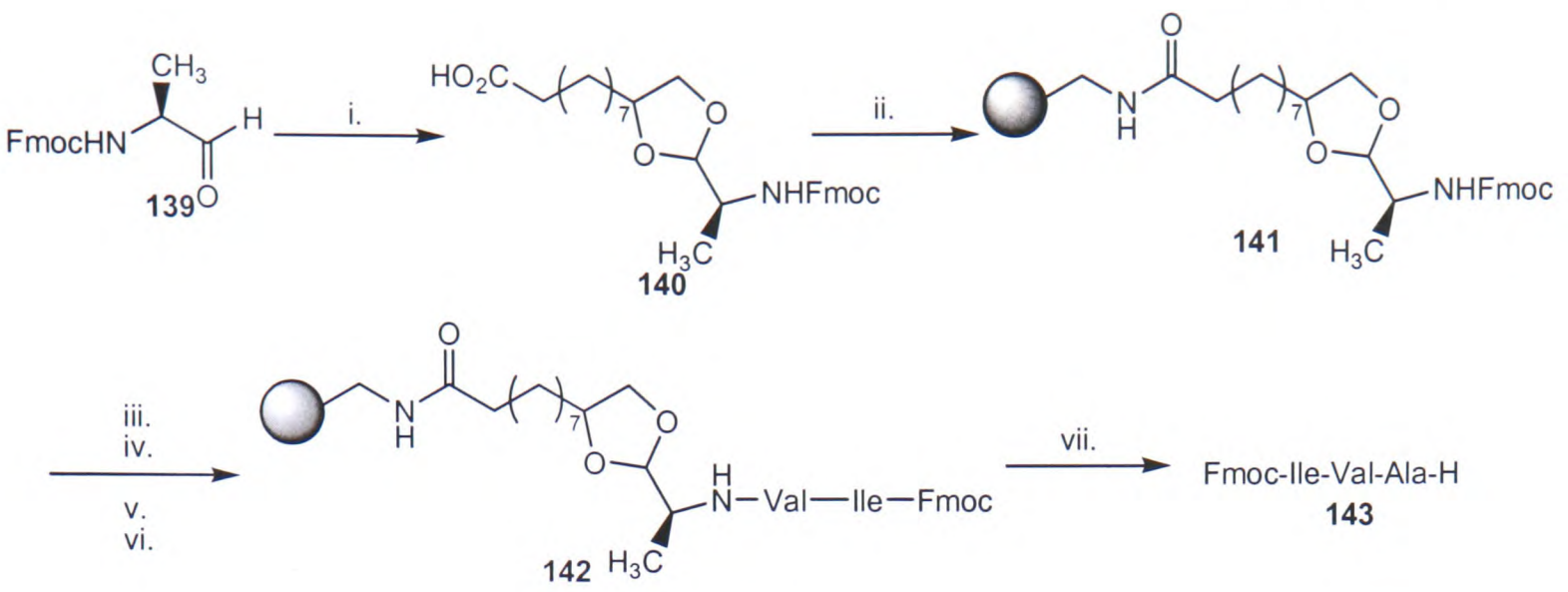
Alaninal acetal **129** was coupled to polystyrene based resin **130** by reductive amination to afford **131**. Peptide elongation was carried out by acylation of the secondary BAL-linked amine **131** using either Fmoc-amino acid as preformed symmetrical anhydrides or the Fmoc-amino acid activated *in situ* by HATU/diisopropylethylamine. Further chain elongation followed standard Fmoc procedures. Treatment of **132** with trifluoroacetic acid resulted in concomitant acetal deprotection and peptide aldehyde, **133** liberation from the solid support. The cleaved peptide aldehyde **133** was found to be a diastereomeric mixture. It was unclear at which stage in the process the racemization occurred.

In 2000, Tam *et. al.*[77] described the synthesis of peptide aldehydes through a cyclic acetal resin **135** (Scheme 36). Fmoc-3-amino-1,2-propanediol was coupled to benzaldehyde- polystyrene **134** in the presence of catalytic *p*-toluenesulfonic acid. After peptide elongation using standard Fmoc chemistry, peptide acetal **137** was released by acid hydrolysis and periodate oxidation generated the peptide aldehyde **138** in quantitative yield. This method offers the possibility of recycling the benzaldehyde-polystyrene resin **134**, therefore reducing the cost of the method.



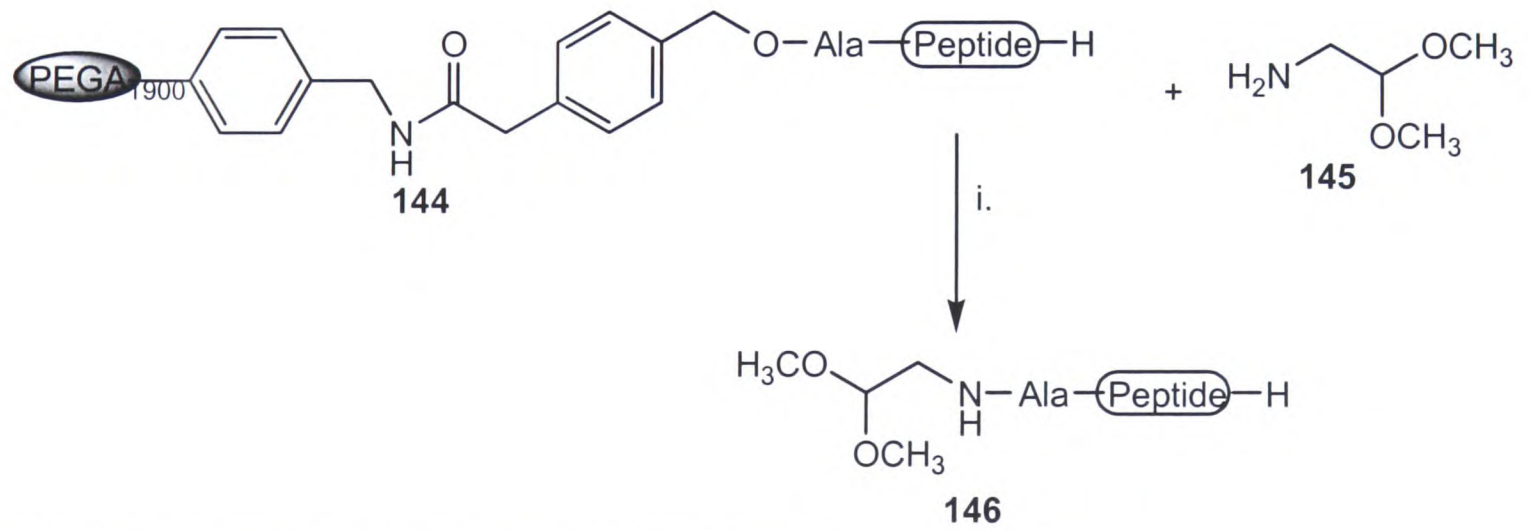
Scheme 36: *Reagents and conditions*: i. $FmocNHCH_2CHOHCH_2OH$, *p*-TsOH, 100 °C, 24 h; ii. 20% piperidine/DMF; iii. Fmoc-AA-OH, DIC, HOBt; iv. Deprotection and coupling; v. TFA; vi. $NaIO_4$, 20% acetic acid, 2 min.

In a slightly different approach, Yao and Xu[78] have prepared peptide aldehydes directly on acetal resin (Scheme 37). This was achieved by treatment of Fmoc-alaninal **139** with *bis*-trimethylsilyl ether to give the acetal **140** after purification by flash chromatography. Acetal **140** was coupled to aminomethylated resin using BOP, HOBt and DIPEA coupling conditions. Elongation by classical Fmoc-synthesis provided the peptide acetal **142** on solid support. Various cleavage conditions were examined for the removal of peptide aldehyde **143**: acid (50% TFA/DCM or 95% TFA/H_2O), temperature (50 °C or room temperature), and reaction time (from 5 min to 12 h). The optimum cleavage conditions were 95% TFA/H_2O, three times over 15 min, at room temperature. Peptide aldehydes **143** were obtained in high yield, purity and stereochemical integrity.



Scheme 37: *Reagents and conditions:* i. TMSOTf, 0 °C; ii. polymer-CH_2NH_2, BOP, HOBt, DIPEA; iii. 20% piperidine/DMF; vi. Fmoc-Val-OH, BOP, HOBt, DIPEA; v. 20% piperidine/DMF; vi. Fmoc-Ile-OH, BOP, HOBt, DIPEA; vii. 95% TFA/H_2O, 3 x 15 min.

Recently, Lelièvre *et. al.*[79,80] reported the synthesis of large peptide aldehydes (33-mer) from solid support through nucleophilic displacement (Scheme 38). Aminolysis of the ester bond between the peptide and phenylacetamidomethyl (PAM) resin **144** led to a peptide aldehyde **146** masked as an acetal, which could be converted to aldehyde by acid hydrolysis. Alanine was chosen as the amino acid attached to the PAM resin to minimise steric hindrance between the aminoacetaldehyde-dimethylacetal **145** and the resin **144** in the nucleophilic displacement.



Scheme 38: *Reagents and conditions:* i. DMF, 18 h, 40 °C.

This report studied a variety of parameters for optimum cleavage of the peptide acetal **146** from solid support including; solvent, temperature, peptide length, workup procedure and the nature of the polymer matrix for optimum cleavage of the peptide acetal from solid support. It was found that a polar matrix for nucleophilic

displacement improved cleavage yields since the matrix was compatible with the unprotected amino acid residues of the peptide chain. Among the solid supports tested (polystyrene, NovaGel, TentaGel and PEGA), PEGA resin afforded the best cleavage yield. This finding illustrates that the solid support should be considered as a co-solvent rather than an inert carrier.

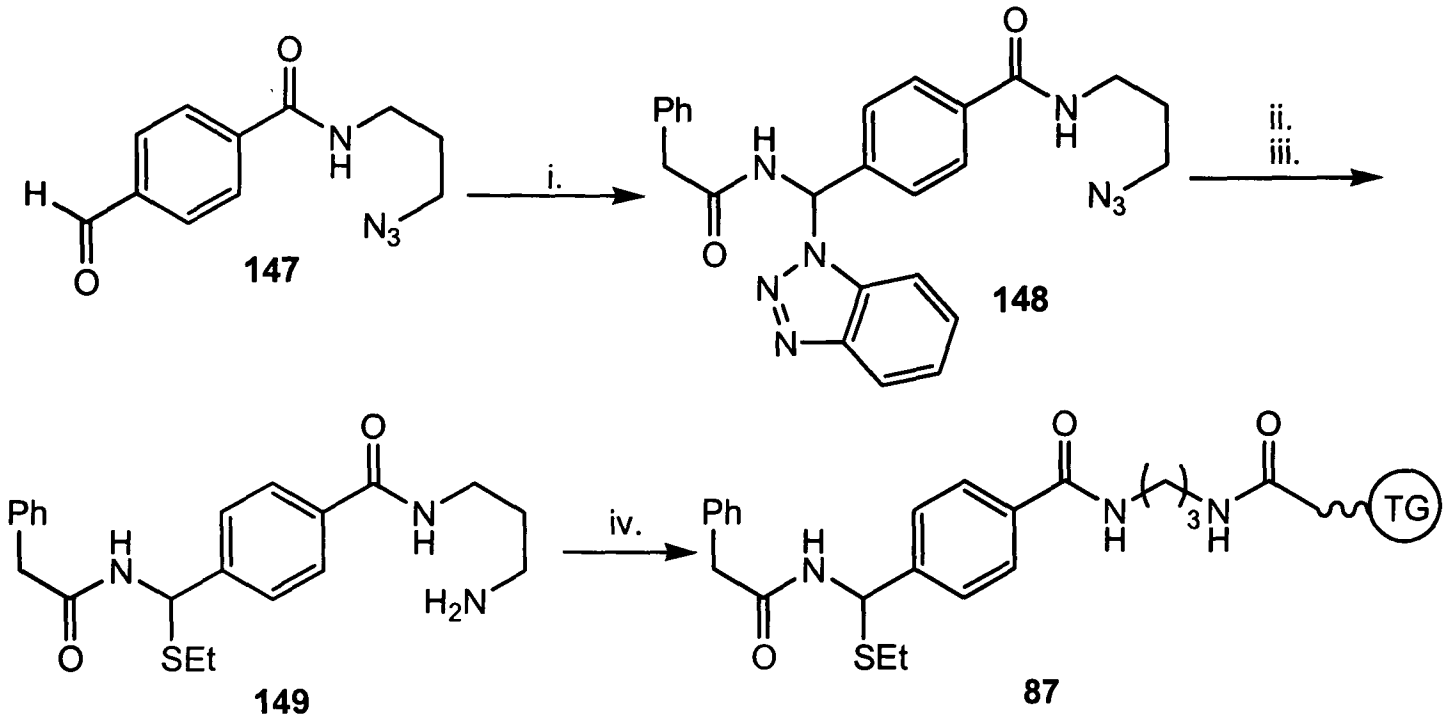
1.3.2 Summary of solid-phase peptide aldehyde synthesis

The demand for linker groups, which are stable under a variety of reaction conditions and allow release of desired products from polymer supports under very mild conditions, has increased with the advent of combinatorial and parallel synthesis of compound libraries. From the previous review, it can be seen that there are several methods for the solid phase synthesis of peptide aldehydes from solid support which can be cleaved using chemical methods;[64,65,67,68,71,73,75,76,78] however, such conditions often limit the applicability of the method. Enzymatic methods have been successfully employed in organic synthesis for a multitude of transformations with explicit chemo-, regio- and stereoselectivity. In principle, the use of enzymatic transformations opens up alternative strategies for the cleavage of aldehydes, namely peptide aldehydes, from solid support.

1.4 Aims of project

The aim of the project was to develop a suitable linker for the synthesis of peptide aldehydes on solid support, which could be cleaved using enzymatic hydrolysis. As mentioned previously, work carried out by Flitsch *et. al.*[50] focussed on a linker **87** suitable for the immobilisation of alcohols and amines (Scheme 39). Linker **87** was constructed using Katritzky[81] methodology by condensation of an aldehyde **147**, benzotriazole and phenylacetamide with azeotropic removal of water. Displacement of benzotriazole with a thioethyl group was carried out to facilitate attachment of alcohols. Thus, treatment of **148** with sodium ethanethiolate followed

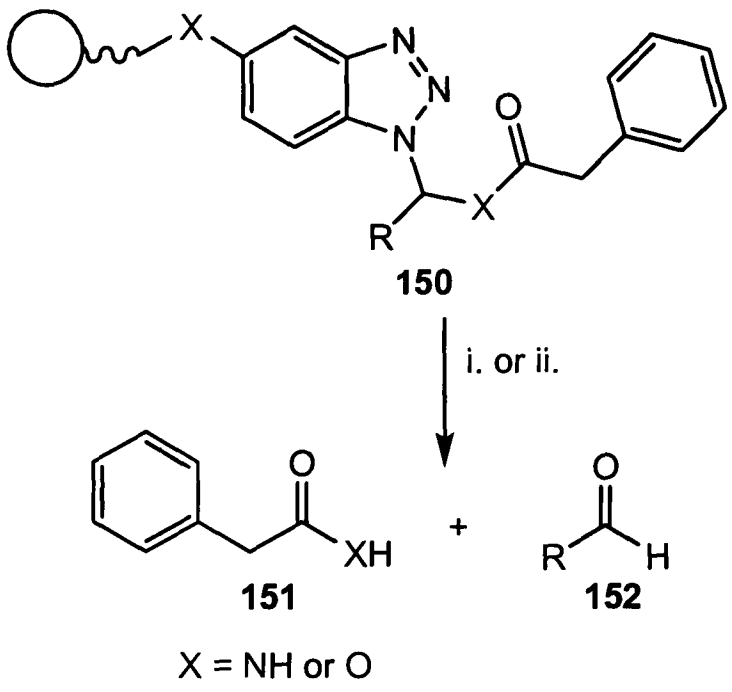
by reduction of the azide group afforded **149**. Amine **149** was coupled to carboxymethyl-TentaGel, using well-known coupling procedures, to give **87**.



Scheme 39: *Reagents and conditions:* i. phenylacetamide, benzotriazole, TsOH, toluene, reflux, Dean-Stark, 16 h, 67%; ii. sodium ethanethiolate, THF, r.t., 4 h, 96%; iii. triphenylphosphine, H_2O, THF, r.t., 16 h, 96%; iv.carboxymethyl-TentaGel, TBTU, HOBt, DIPEA, DMF, 16 h, 25 °C.

Linker **87** can be cleaved using mild acid or base hydrolysis; in addition to, enzymatic hydrolysis with penicillin acylase (50% cleavage). The utility of linker **87** was demonstrated by the synthesis of glycopeptides.[82]

It is envisaged that aminal linker **87** could be extended to allow for the synthesis of peptide aldehydes **152** on solid support by simply tethering the linker **87** to solid support through a benzotriazole moiety to give aminal linker **150** (Scheme 40). Subsequent, peptide elongation followed by treatment with mild acid/base or penicillin acylase would release the desired peptide aldehyde **152** and phenylacetic acid/phenylacetamide **151**.



Scheme 40: *Reagents and conditions:* i. acid or base; ii. penicillin acylase.

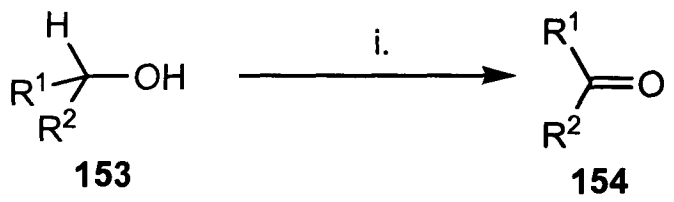
2 Results and discussion – Synthesis of amino aldehydes

2.1 Reported methods for the synthesis of amino aldehydes

In order to construct the aldehyde linker **150** using Katritzky methodology[81] (Scheme 40), a suitable method for the solution synthesis of amino aldehydes was required. A recent review by Jurczak *et. al.*[83] highlighted the methods available for the preparation of amino aldehydes.

2.1.1 Oxidative methods for the preparation of amino aldehydes.

The most commonly used method for the synthesis of aldehydes is the oxidation of alcohols.[84] In 1978, Omura and Swern[85] reported the use of dimethylsulfoxide, activated with oxalyl chloride, as an oxidant for alcohols **153** (Scheme 41). Despite the problems associated with this method, including the production of dimethylsulfide (stench) and toxic gaseous side products (phosgene and carbon monoxide), this method has gained increasing importance. There are many reasons for the popularity of this reaction and these include the use of mild conditions, volatile by-products and lack of over-oxidation to carboxylic acids. This method has become a well established procedure for the oxidation of primary, secondary, allylic, benzylic, hindered and bicyclic alcohols.[86]

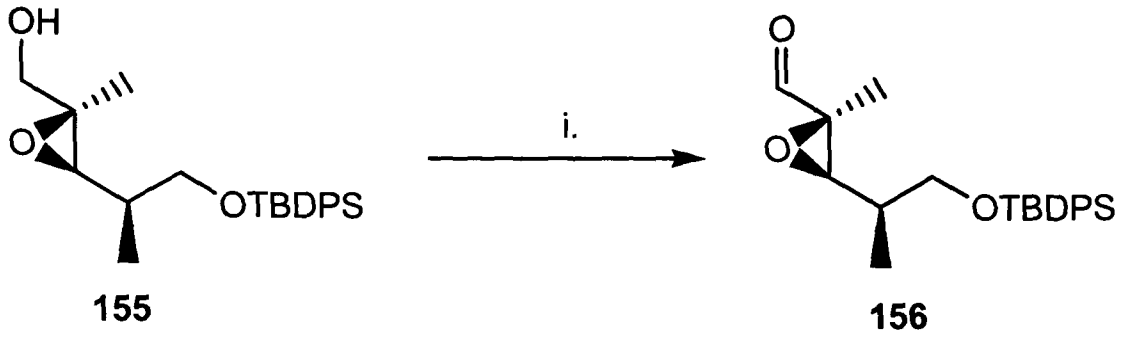


153 **154**

Scheme 41: *Reagents and conditions:* i. oxalyl chloride, dimethylsulfoxide, anhydrous dichloromethane, triethylamine, -60 °C.

The use of tetrapropylammonium perruthenate, TPAP was first introduced in 1987 by Griffith and Ley.[87] TPAP is a commercially available, organic soluble oxidant which has been used to effect oxidation in complex molecules without disturbing other functional groups (Scheme 42).[88] Aldehydes bearing a labile α-stereogenic centre can be prepared without scrambling of stereochemistry. One

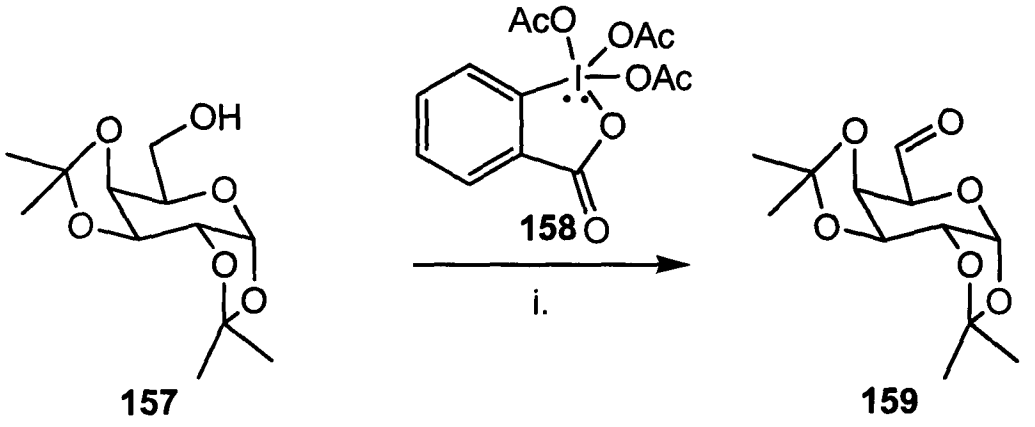
advantage of TPAP is that it can be used as a catalyst in conjunction with an excess of *N*-methylmorpholine-*N*-oxide (NMO). Hinzen and Ley[89] have developed a polymer supported perruthenate reagent that can be used in the same way as TPAP for the oxidation of alcohols.



155 **156**

Scheme 42: *Reagents and Conditions:* i. TPAP, NMO, 4 Å molecular sieves, dichloromethane, r.t., Ar(g), 1 h, 70%.

In 1983, Dess and Martin[90] discovered that 1-hydroxy-1,2-benziodoxol-3(1*H*)-one, IBX could be transformed into the more soluble periodinane, 1,1,1-triacetoxy-1,1-dihydro-1,2-benziodoxol-3(1*H*)-one **158**. Since then, the Dess-Martin periodinane **158** has been used for the selective transformation of alcohols **157** into aldehydes **159**[91] (Scheme 43).



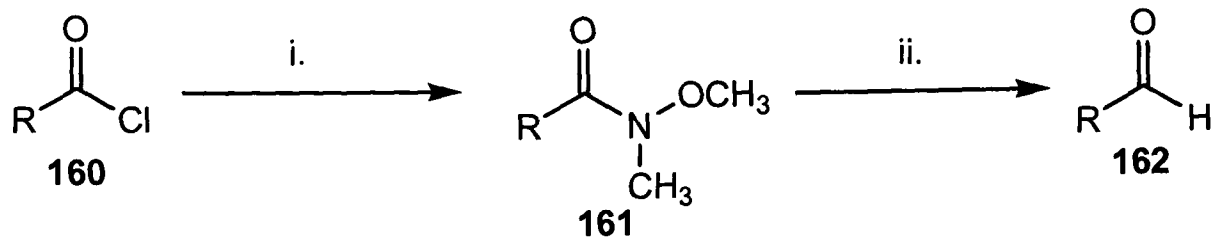
157 **159**

Scheme 43: *Reagents and conditions:* i. dichloromethane, 12 h, 58%.

2.1.2 Reductive methods for the preparation of amino aldehydes.

Another useful method for the synthesis of aldehydes is *via* Weinreb amides **161**.[66] These are prepared from commercially available *N,O*-dimethylhydroxylamine and the corresponding acid chloride **160** or other activated derivative. Reduction of **161** with lithium aluminium hydride furnishes the desired aldehyde **162** (Scheme 44). This method has gained popularity due to the fact that over-reduction and

racemization of the α-stereocentre do not occur. *C*-terminal peptide aldehydes have been prepared using this method.[92]



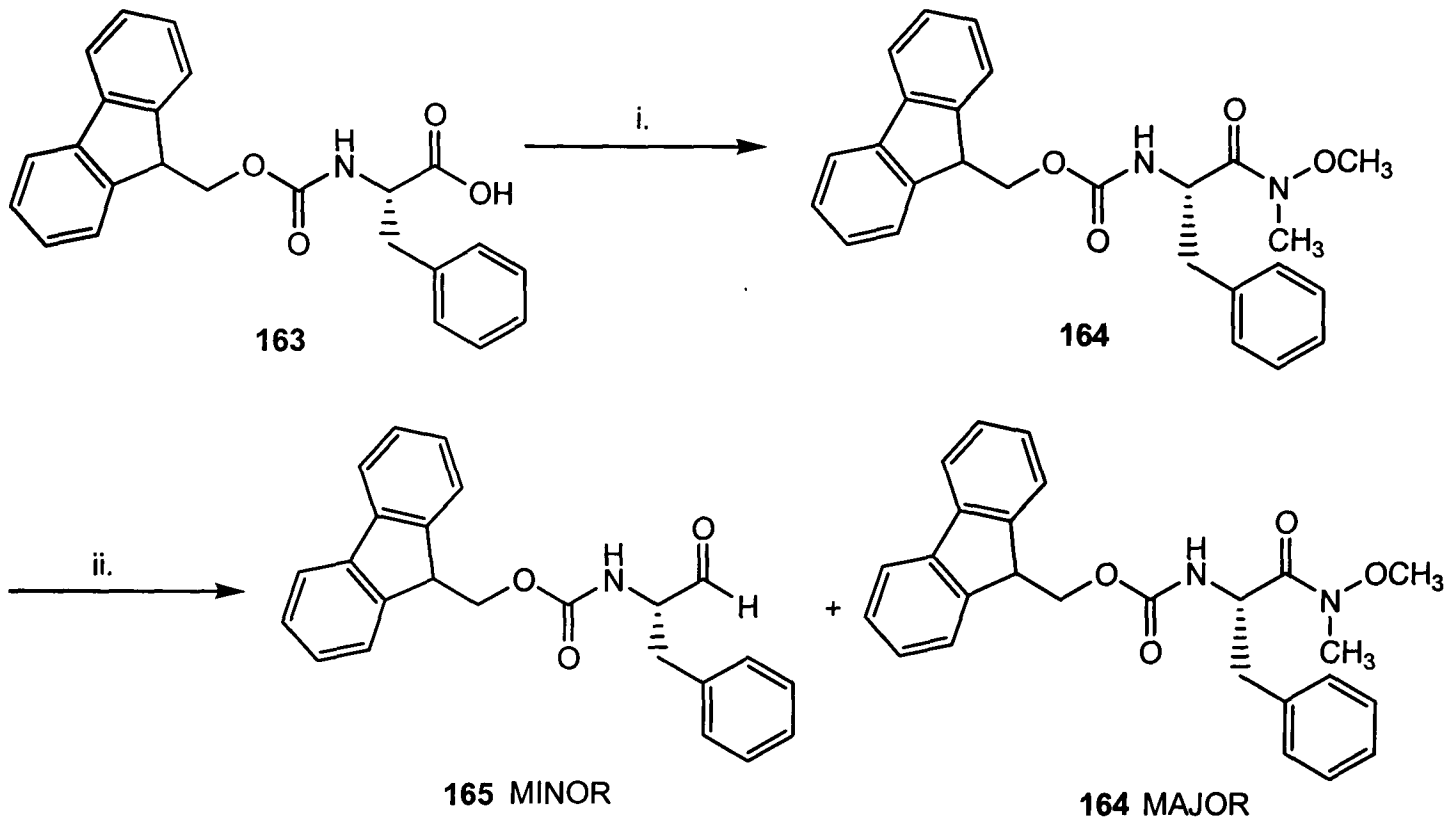
Scheme 44: *Reagents and conditions:* i. *N,O*-dimethylhydroxylamine hydrochloride, $CHCl_3$, pyridine, 0 °C; ii. $LiAlH_4$, THF, -78 °C, N_2.

2.2 Attempted synthesis of 9-fluorenylmethoxycarbonyl-phenylalaninal

Since it was the intention to tether the protected amino aldehyde to a solid support, it was crucial that the amine protecting group was compatible with the conditions required for solid phase synthesis. That is, the protecting group must be easily removed at the end of the synthesis using mild conditions, which have no adverse effects on the rest of the structure. *N*-Fluorenylmethoxycarbonyl (Fmoc) is one of the most common protecting groups in solid phase chemistry. Generally, the Fmoc group is relatively stable under acidic conditions, but can be easily removed under basic conditions (20% piperidine in DMF). Fmoc-protected amino acids are cheap, commercially available compounds making them ideal as possible starting materials for the synthesis of amino aldehydes.

The first method attempted for the synthesis of amino aldehydes was *via* reduction of Weinreb amides. (*S*)-Fmoc-phenylalanine **163** was readily converted to the corresponding Weinreb amide **164** in good yield (80%), by coupling with *N,O*-dimethylhydroxylamine hydrochloride in the presence of base using HBTU as a coupling reagent. Subsequent reduction of **164** using lithium aluminium hydride in tetrahydrofuran (THF) afforded only small amounts of desired aldehyde **165** (Scheme 45). This suggested that the stabilised tetrahedral intermediate formed by coordination of the methoxy group to the metal, was not being suitably quenched in acidic $KHSO_4$ (HSO_4^-; $pK_a = 1.92$). In hindsight, a stronger acid (*e.g.* H_2SO_4; $pK_a = -3$) might facilitate complete conversion to the aldehyde **165**. Several other Fmoc-

Weinreb amides (*i.e.* glycine, valine, alanine and leucine) were reduced using the same method; however this yielded limited success. In some cases, the loss of the Fmoc protecting group was observed.



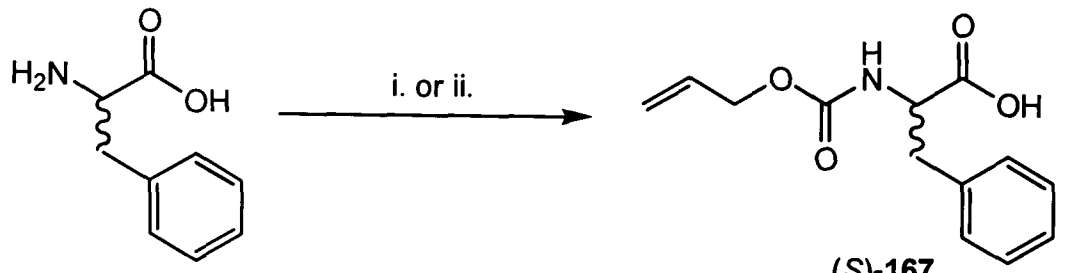
Scheme 45: *Reagents and conditions:* i. *N,O*-dimethylhydroxylamine hydrochloride, diisopropylethylamine, HBTU, dichloromethane, 1 h, r.t., 80%. ii. $LiAlH_4$, THF, -20 °C, 50 min.

2.3 Synthesis of allyloxycarbonyl-phenylalaninal *via* a Weinreb amide.

Since the Fmoc group appeared to be unstable, a more durable protecting group was sought. The allyloxycarbonyl (Alloc) group is sufficiently robust to withstand the basic conditions of the removal of Fmoc groups and the acidic conditions of Boc removal.[93] However, it can be removed from the solid phase with tetra*kis*(triphenylphosphine)palladium (0), in the presence of a scavenger. The scavenger prevents re-addition of the allyl unit onto the amine. Various scavengers have been used including dimedone[94] and amine/borane complexes.[95]

There are two main reagents used for *N*-Alloc protection: allylchloroformate or diallyl pyrocarbonate. The latter does not require an additional base and the only by-products, carbon dioxide and allyl alcohol, are volatile. Both methods were used, depending on which starting materials were available at the time.

(*S*)-(-)-Phenylalanine **166** was protected using allylchloroformate in the presence of 4M sodium hydroxide (86%) or diallyl pyrocarbonate at reflux to give **167** (61%) (Scheme 46). The former method was used to prepare racemic Alloc-protected phenylalanine **169** in good yield (80%). The amino acids, **167** and **169** were obtained in sufficient purity to be used without further purification.

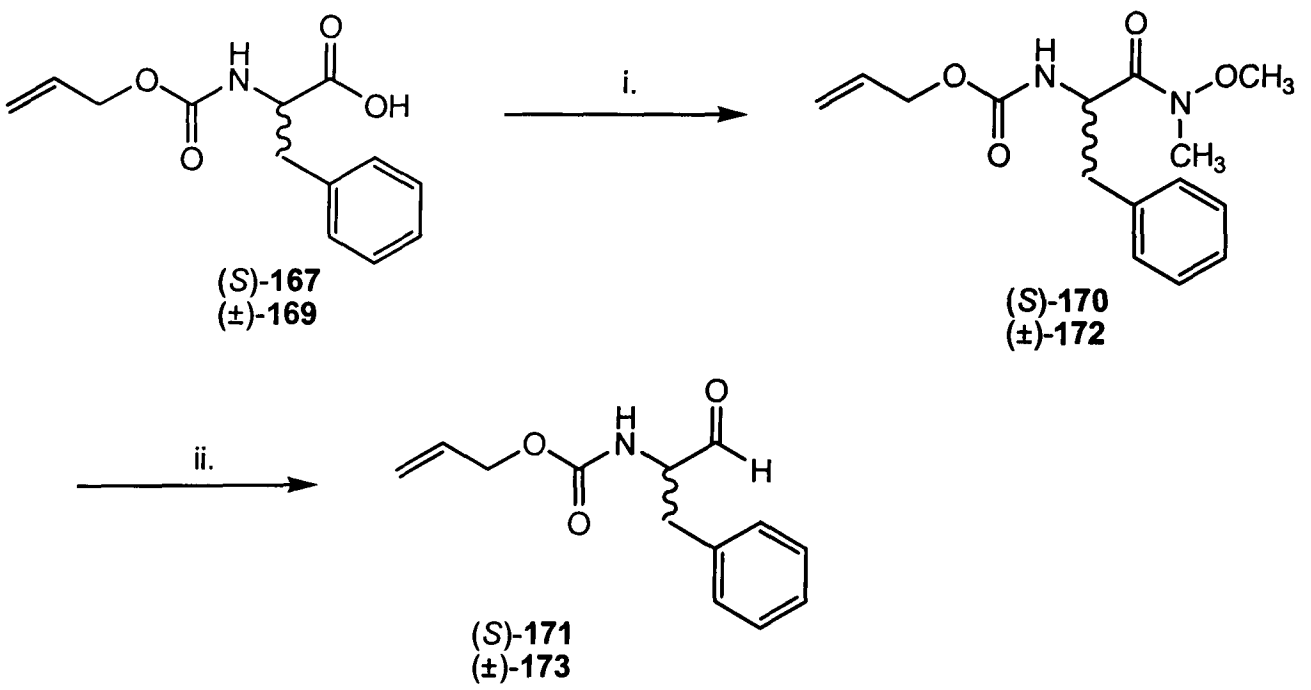


(*S*)-**166**
(±)-**168**

(*S*)-**167**
(±)-**169**

Scheme 46: *Reagents and conditions:* i. allyl chloroformate, 4 M NaOH, 0 °C for 15 min, then r.t for 15 min, ii. diallyl pyrocarbonate, H_2O/dioxane (3:2), reflux, 30 min.

170 was prepared using the same method as Fmoc-amino acid **164** (Section 2.2) (Scheme 47). Chiral HPLC (Section 8.2.9) confirmed the enantiomeric purity of the Weinreb amide (>99% e.e.). Aldehyde **171** was generated by reduction of Weinreb amide **170** with lithium aluminium hydride. The crude aldehyde **171** was used without further purification, since silica gel is known to epimerize α-amino aldehydes[83]. The chiral integrity (98% e.e.) of the aldehyde **171** was confirmed by reduction to the corresponding alcohol with sodium borohydride (Section 8.3.18), followed by chiral HPLC (Section 8.2.9). Racemic Alloc-protected phenylalaninal **173** was prepared using the same method.



(*S*)-**167**
(±)-**169**

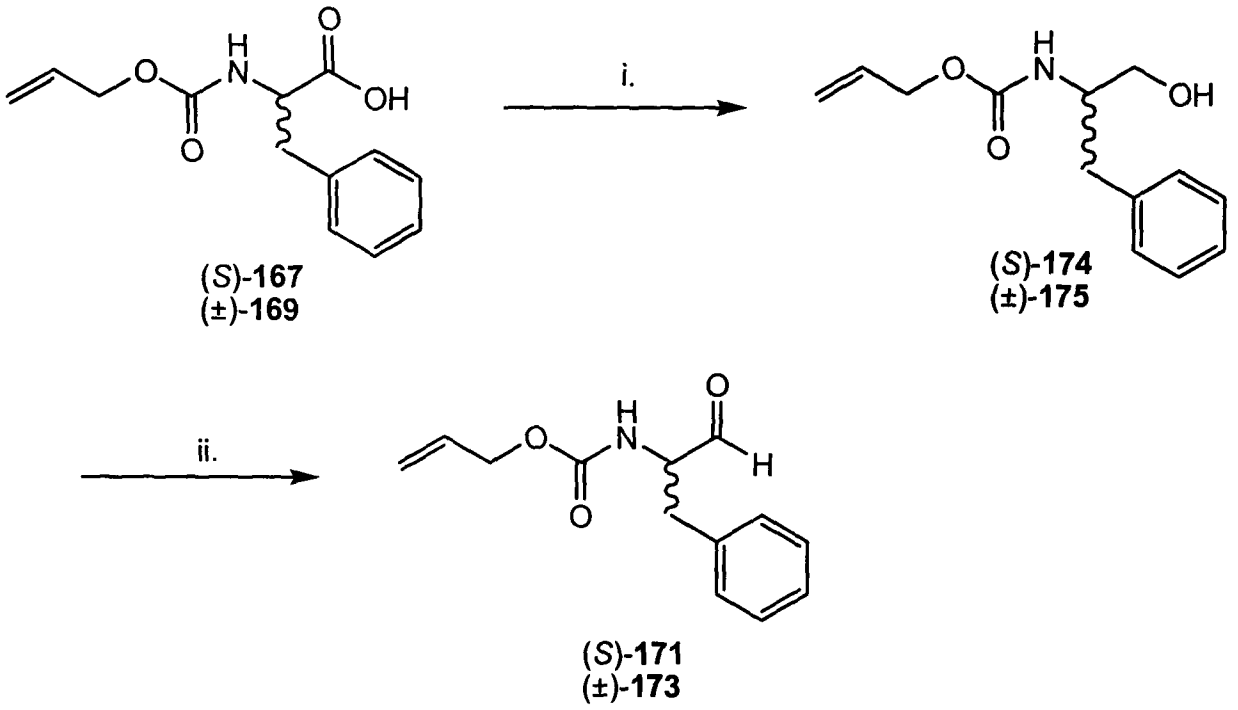
(*S*)-**170**
(±)-**172**

(*S*)-**171**
(±)-**173**

Scheme 47: *Reagents and conditions:* i. *N,O*-dimethylhydroxylamine **hydrochloride,** diisopropylethylamine, HBTU, dichloromethane, 1 h, r.t. ii. $LiAlH_4$, THF, -20 °C, 50 **min.**

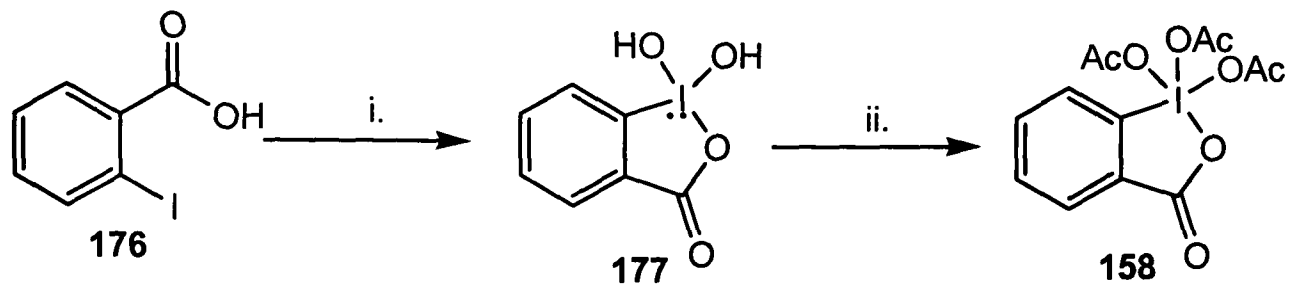
2.4 Synthesis of allyloxycarbonyl-phenylalaninal *via* oxidation of alcohol

In an alternative strategy, (*S*)-Alloc-phenylalaninol **174** was generated by reacting (*S*)-Alloc-phenylalanine **167** with isobutylchloroformate to form the mixed anhydride, followed by reduction with sodium borohydride to give the alcohol **174**[96] (33% yield, 98% e.e. by chiral HPLC (Section 8.2.9) (Scheme 48). The alcohol was used without further purification. This procedure was repeated for the preparation of racemic Alloc-phenylalaninol **175**.



Scheme 48: *Reagents and conditions:* i. isobutyl chloroformate, *N*-methylmorpholine, 1,2-dimethoxyethane, -15 °C, then $NaBH_4$ in H_2O. ii. Dess-Martin Periodinane, water-saturated dichloromethane, r.t., 40 min.

IBX, 1-hydroxy-1,2-benziodoxol-3(1*H*)-one **177** was prepared from 2-iodobenzoic acid **176** by oxidation with oxone ($2KHSO_5$-$KHSO_4$-K_2SO_4)[97] (Scheme 49). Dess-Martin periodinane **158** was obtained using the modified Ireland[98] procedure, in which **177** was treated with acetic anhydride in catalytic *p*-toluene sulfonic acid to afford the periodinane **158** in good yield (80%). Treatment of (*S*)-Alloc-phenylalaninol **174** with Dess-Martin periodinane **158** in water-saturated dichloromethane[99] afforded the desired aldehyde **171** in good yield (79%) and >99% e.e., as determined by reduction with sodium borohydride (Section 8.3.18) followed by chiral HPLC (Section 8.2.9). Again, racemic Alloc-phenylalaninal **173** was produced in reasonable yield (61%), using the Dess-Martin periodinane **158** oxidation method.



Scheme 49: *Reagents and conditions:* i. oxone, H_2O, 70 °C, 1 h, 96%; ii. acetic anhydride, *p*-TsOH, 80 °C, 2 h, 80%.

2.5 Summary and conclusions

Initial attempts to synthesize Fmoc-protected amino aldehydes **165** *via* Weinreb amides **164** were hampered by incomplete conversion to the aldehyde **165** and in some cases, loss of the Fmoc protecting group occurred (Scheme 45). These problems were overcome by the use of allyloxycarbonyl as a protecting group. (*S*)-Allyloxycarbonyl-phenylalaninal **171** was prepared in good yield and chiral purity using two methods:

1. *via* a Weinreb amide **170** (76% overall yield, 98% e.e.).
2. *via* oxidation of the corresponding alcohol **174** (56% overall yield, >99% e.e.).

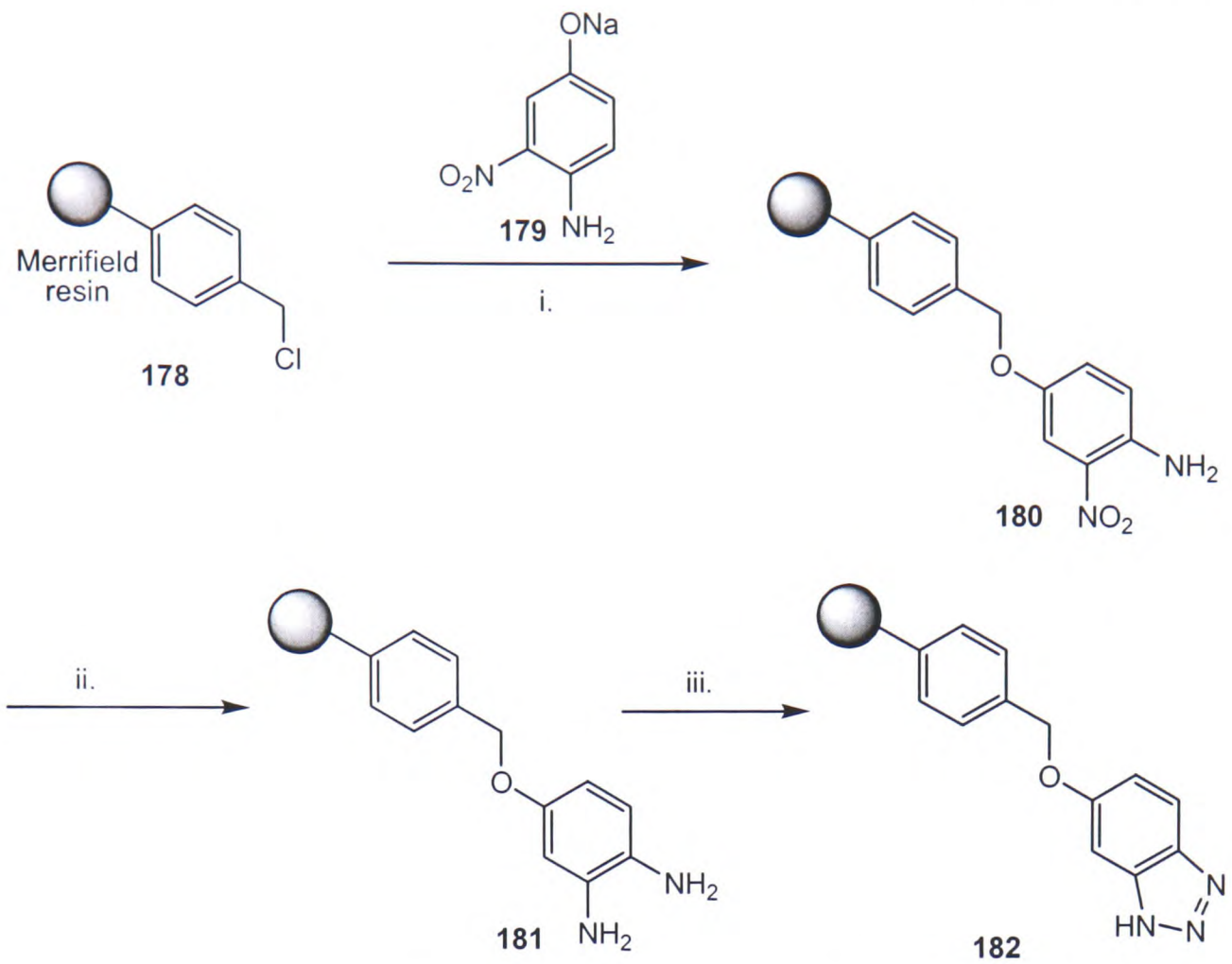
Racemic allyloxycarbonyl-phenylalaninal **173** was also prepared in good yield (72-86% overall yield) using both methods, 1. and 2.

3 Results and discussion - Synthesis of 1*H*-benzotriazole resins

To allow for the attachment of the allyloxycarbonyl-phenylalaninal **171/173** to a solid support, an efficient method for the preparation of polymer bound 1*H*-benzotriazole was essential.

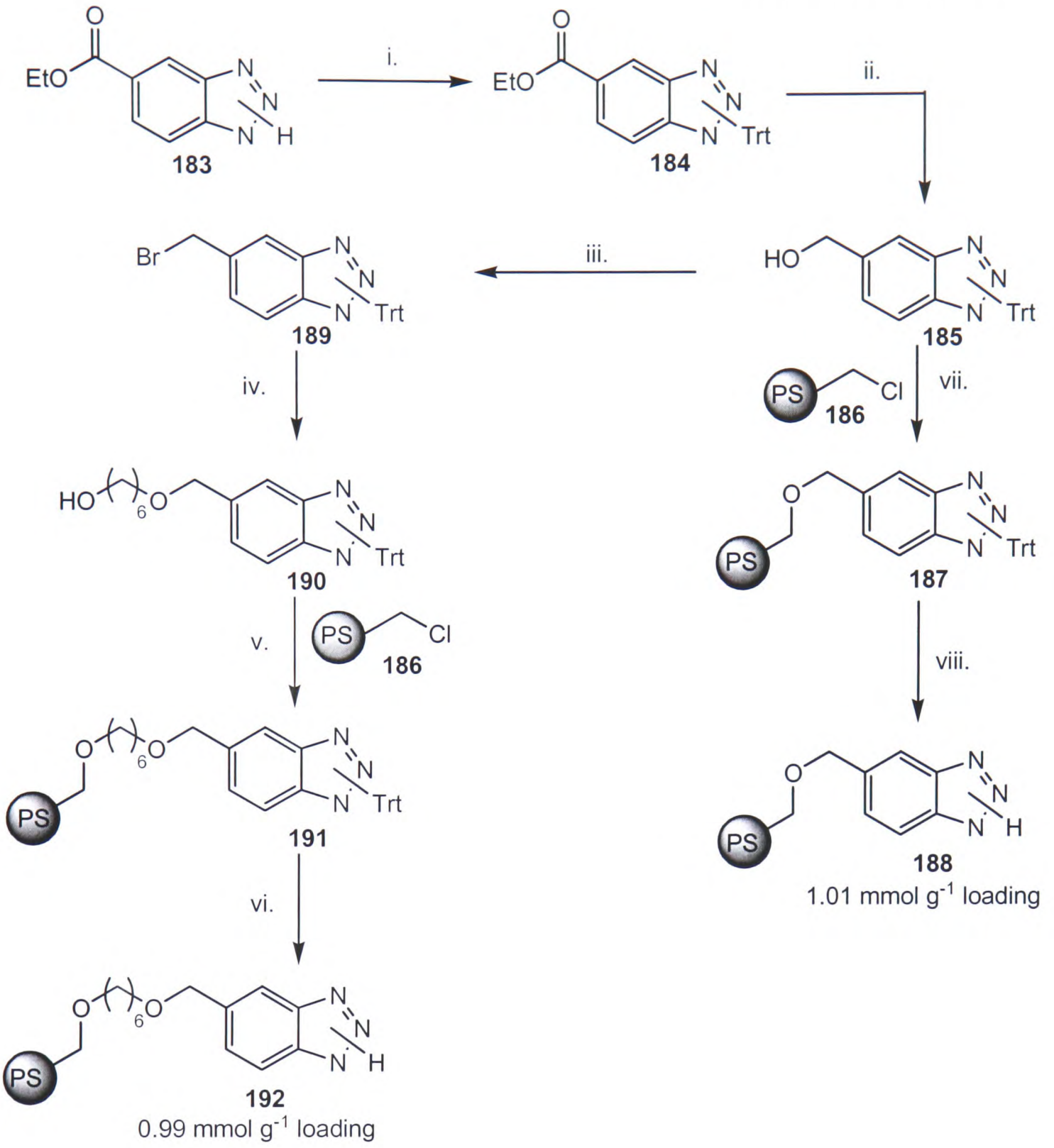
3.1 Reported syntheses of polymer bound 1*H*-benzotriazole

In 1999, Katritzky *et. al.*[100] published the first preparation of solid-phase bound 1*H*-benzotriazole. Treatment of Merrifield resin **178** with sodium salt **179** (Scheme 50) afforded the corresponding resin-bound nitroaniline **180**. The nitro group was reduced using stannous chloride to give **181**. Subsequent treatment of **181** with isoamyl nitrite afforded the benzylether-1*H*-benzotriazole resin **182**. The gel phase ^{13}C NMR spectrum of **182** was unresolved, even after lengthy acquisition. However, the FTIR spectrum of **182** showed a very broad band (2700-3400 cm^{-1}), which was attributed to the NH group of the hetero ring (broadening is explained by the equilibrium between the 1*H*, 2*H* and 3*H*-isomers in the benzotriazole backbone. The NH bond in each isomer varies in length and strength and will therefore show a range of stretching frequencies distributed about a mean value).



Scheme 50: *Reagents and conditions:* i. *N,N*-dimethylacetamide (DMA), 80 °C, 6 h; ii. stannous chloride, DMA, 60 °C, 5 h, then r.t for ~16 h; iii. Dioxane, acetic acid, HCl, isoamyl nitrite, r.t., 24 h, then 50-60 °C for 0.5 h.

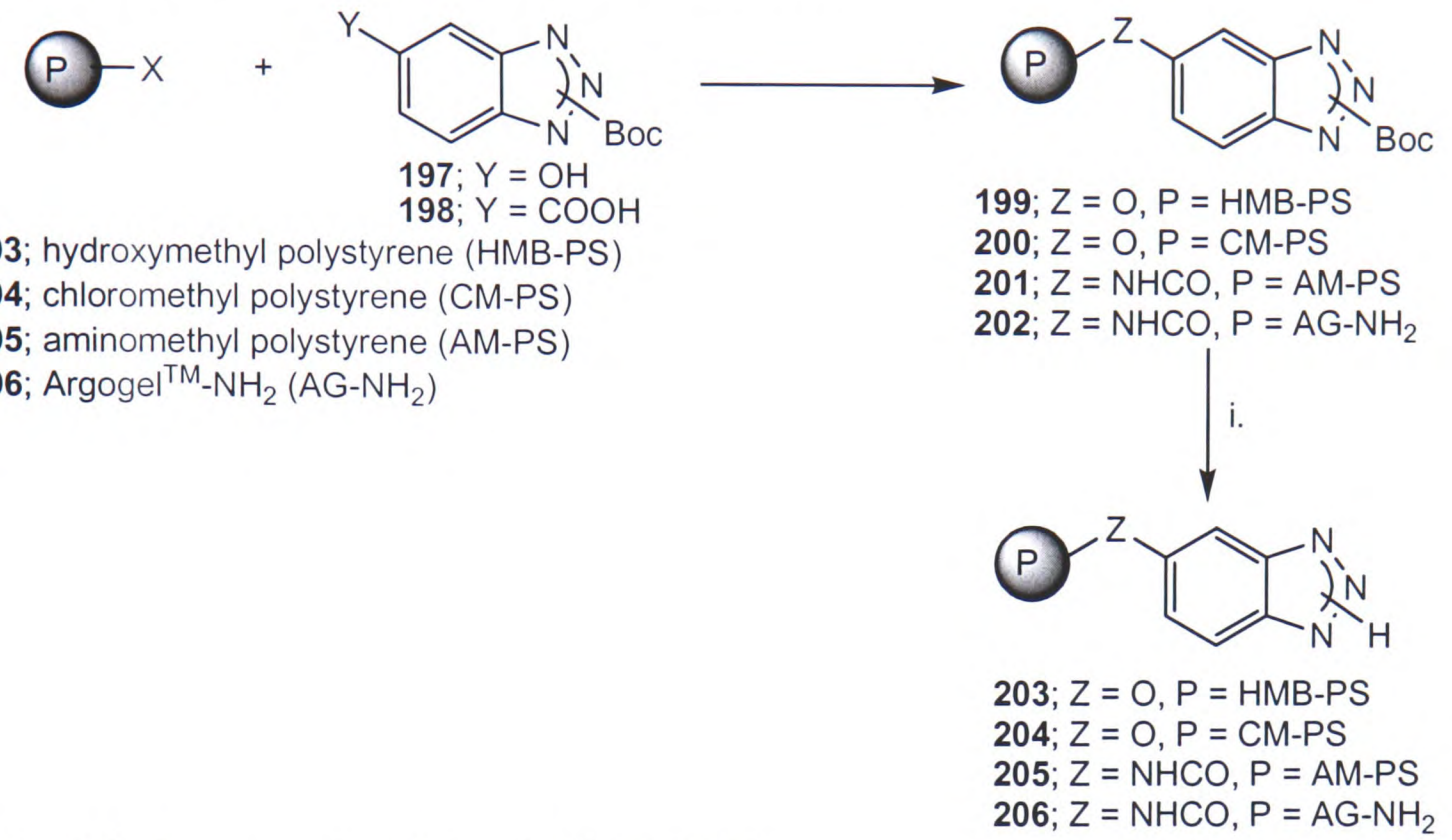
Longer, more flexible linkers for the attachment of benzotriazole were investigated by Schiemann and Showalter.[101] Polystyrene-supported benzotriazole **188** and **192** with mono- and diether anchors were prepared (Scheme 51). These linkers **188** and **192** showed sufficient swelling (>7 mL g^{-1} for **192**) in DMF, THF and toluene. The swelling properties can be attributed to the use of hydrophilic ether tethers and lower polymer cross-linking; thus **192** was more accessible to solvation than **188**. However, it was found that resins with better swelling properties did not necessarily improve the reactivity of the benzotriazole auxiliary for Mannich-type reactions.



Scheme 51: *Reagents and conditions:* i. NaH, TrtCl, THF, 95%; ii. $LiAlH_4$, THF, reflux, 98%; iii. CBr_4, PPh_3, DIPEA, THF, 0 °C, 95%; iv. $HO(CH_2)_6OH$, KO*t*-Bu, TBAI, DMSO, 75%, v. KH, TBAI, THF, reflux; vi. HCl, MeOH, THF, 100%; vii. NaH, TBAI, THF, reflux; viii. Conc. HCl, MeOH, THF, 100%.

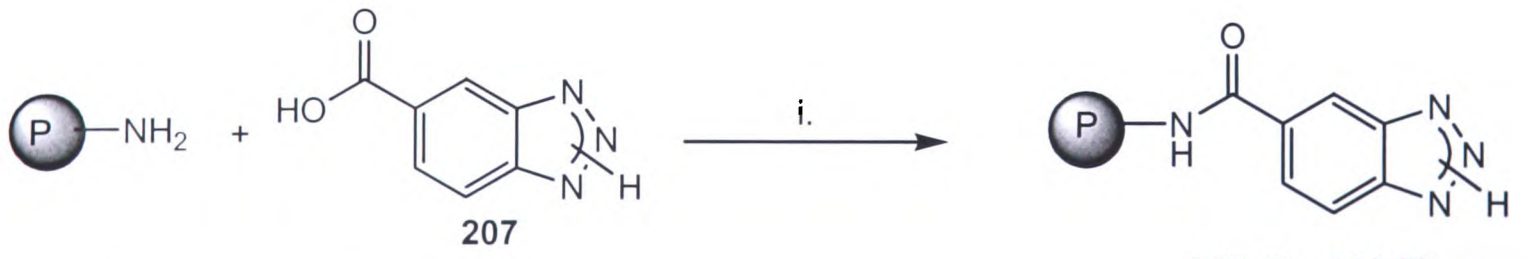
Two papers by Paio *et. al.*[102,103] reported the preparation of 1*H*-benzotriazole resins tethered through both an ether and an amide moiety. In the first paper,[102] *N*-Boc protected 5-hydroxy-benzotriazole **197** was anchored onto hydroxymethyl-polystyrene resin **193** using Mitsunobu solid phase conditions and onto chloromethyl polystyrene **194** using nucleophilic substitution conditions, obtaining compounds **199** and **200** respectively. Compound **198** was tethered onto aminomethyl polystyrene

195 and onto Argogel-NH_2 **196** using conventional peptide coupling conditions to obtain **201** and **202** respectively (Scheme 52). These reactions were monitored by FT-IR. Treatment of **199-202** with acid removed the Boc protecting group to give deprotected polymer bound benzotriazoles **203-206**.



Scheme 52: *Reagents and conditions:* i. 30% TFA/DCM.

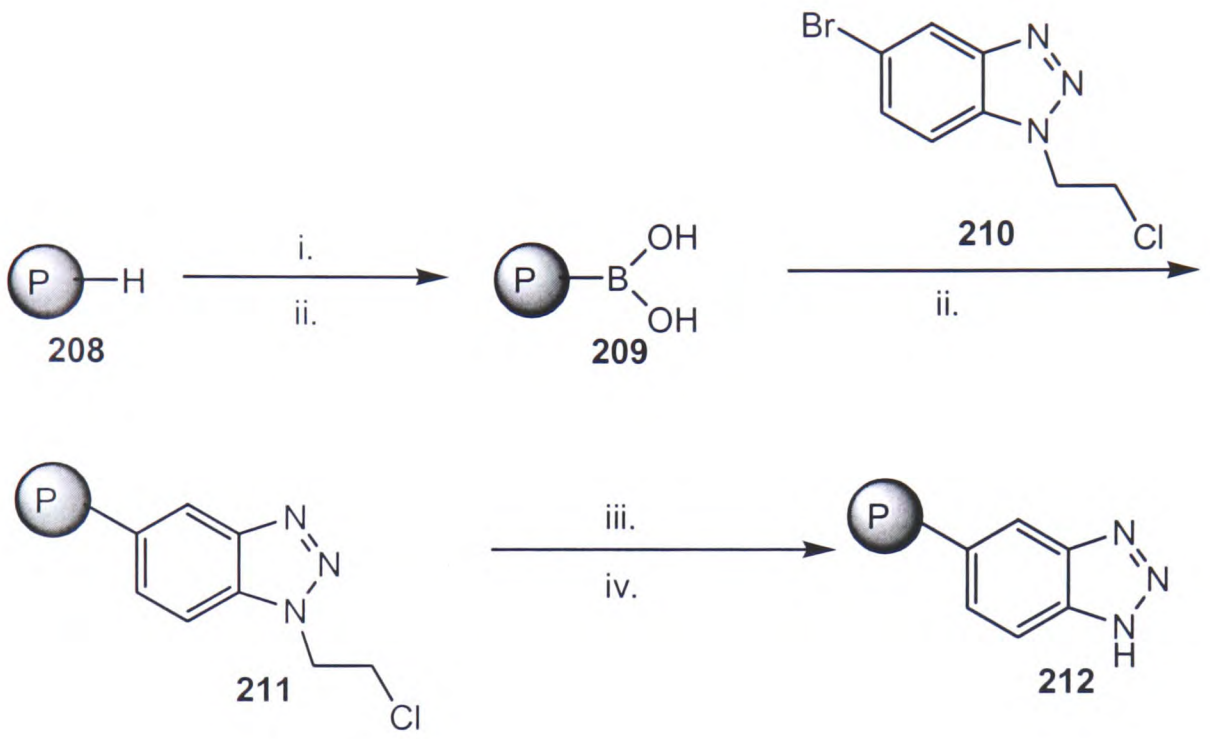
In the later paper,[103] benzotriazole-5-carboxylic acid **207** was bound to polystyrene resins **195** and **196** without the need for protection at the reactive nitrogen (Scheme 53). Commercially available benzotriazole-5-carboxylic acid **207** was reacted with the appropriate polystyrene resin **195** or **196** in the presence of diisopropylcarbodiimide (DIC) and 1-hydroxybenzotriazole (HOBt) in DMF. Quantitative coupling was observed using the colourimetric Kaiser test.[104] Supported derivatives **205** and **206** were characterized by magic angle spinning NMR and FT-IR spectra.



195; aminomethyl polystyrene (AM-PS)
196; Argogel™-NH_2 (AG-NH_2)

Scheme 53: *Reagents and conditions:* i. DIC, HOBt, DMF, r.t., 18 h.

In 2001, Katritzky *et. al.*[105] examined the attachment of benzotriazole directly to a polymer support *via* a carbon-carbon bond (Scheme 54). This employed the use of a Suzuki coupling reaction. Upon treatment of benzotriazole with bromo-chloroethane in the presence of sodium hydride, followed by direct bromination, the desired 5-bromo compound **210** was isolated. Compound **210** was heated with polymer bound boronic acid **209** in the presence of palladium catalyst at 90 °C for 24 h to give polymer bound benzotriazole **211**. Subsequent deprotection afforded **212**.



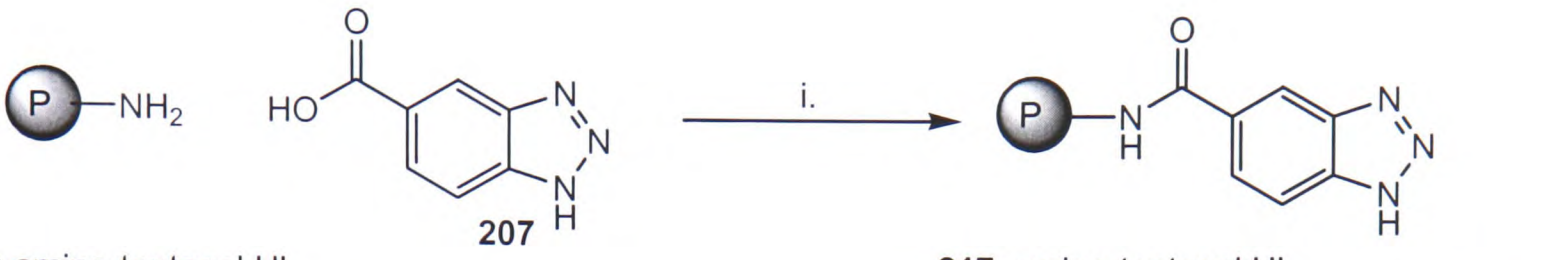
Scheme 54: *Reagents and conditions:* i. Br_2, benzene; ii. BuLi, benzene, $B(OMe)_3$, THF, H^+; iii. $Pd(OAc)_2$, (*o*-Tol)$_3$P, DMF/Et_3N; iv. HCl (6 N), reflux.

3.2 Synthesis of polymer bound 1*H*-benzotriazole adducts

The synthetic scheme outlined (Scheme 53) allows the generation of polymer-bound 1*H*-benzotriazole **205/206** in one step from commercially available benzotriazole-5-carboxylic acid **207**. A range of these adducts on different polymers were prepared to allow for the comparison in physical properties and compatibility with enzyme hydrolysis conditions.

Polymer-bound 1*H*-benzotriazole adducts **217-220** were obtained by reaction of benzotriazole-5-carboxylic acid **207** with the appropriate amino-resin **213-216** in the presence of DIC and HOBt (Scheme 55). Tethering of benzotriazole-5-carboxylic acid **207** to the solid support resulted in the appearance of brown resin beads/gel

which provided a useful visual indication of reaction success. The 2,4,6-trinitrobenzene sulfonic acid (TNBS) test could not be used as a colourimetric test, since the colour of brown resin beads and the red colour of a positive TNBS test were indistinguishable. The Kaiser test (Section 8.2.5.1) confirmed quantitative coupling of **217-220** in all cases.



213; amino-tentagel HL
214; amino-methylated polystyrene HL
215; aminomethyl polystyrene MP
216; $PEGA_{1900}$

217; amino-tentagel HL
218: amino-methylated polystyrene HL
219; aminomethyl polystyrene MP
220; $PEGA_{1900}$

Scheme 55: *Reagents and conditions:* i. DIC, HOBt, DMF, r.t., 16 h.

In most cases, **217-219** the appearance of amide stretches at ~1650 and 1540 cm^{-1} indicated the successful formation of the amide linkage (Figure 10).

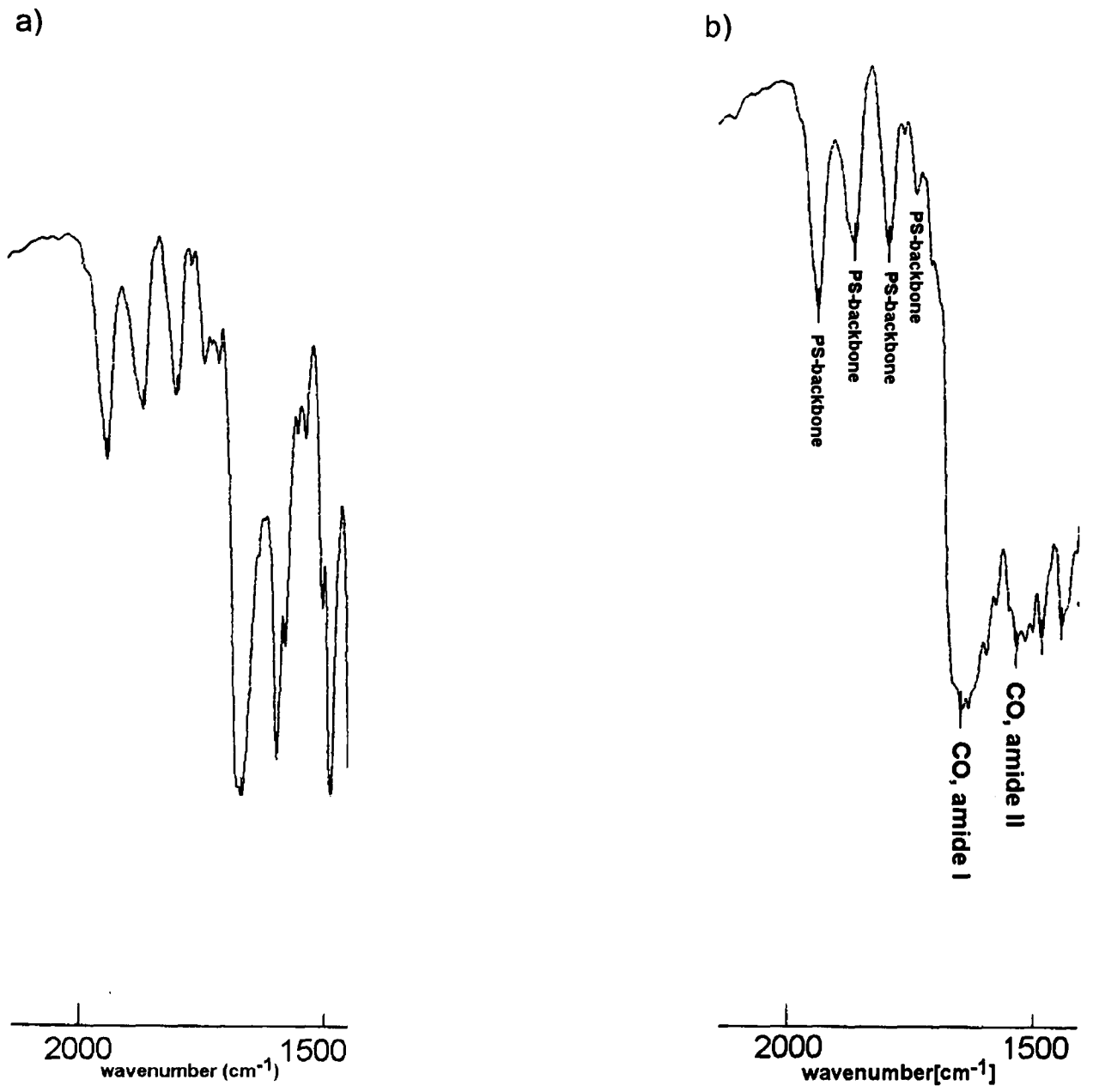


Figure 10: *FT-IR spectrum of a) aminomethylated polystyrene **214** (KBr disc) and b) **218** (KBr disc).*

In the $PEGA_{1900}$ resin **220**, there was overlap of the amide stretches from the polymer backbone and the new amide linkage formed. In this case, loss of the NH_2 stretch at 3621 cm^{-1} in the FT-IR, together with the series of broad signals observed in the aromatic region (7.60-8.50 ppm) of the magic angle spinning (MAS) ^1H NMR spectrum, provided sufficient evidence for polymer bound 1*H*-benzotriazole **220** (Figure 11). In the case of **218**, only one signal from the benzotriazole (7.84 ppm) was apparent in the MAS ^1H NMR spectrum, as the rest of the aromatic signals were masked by the polystyrene aromatic backbone.

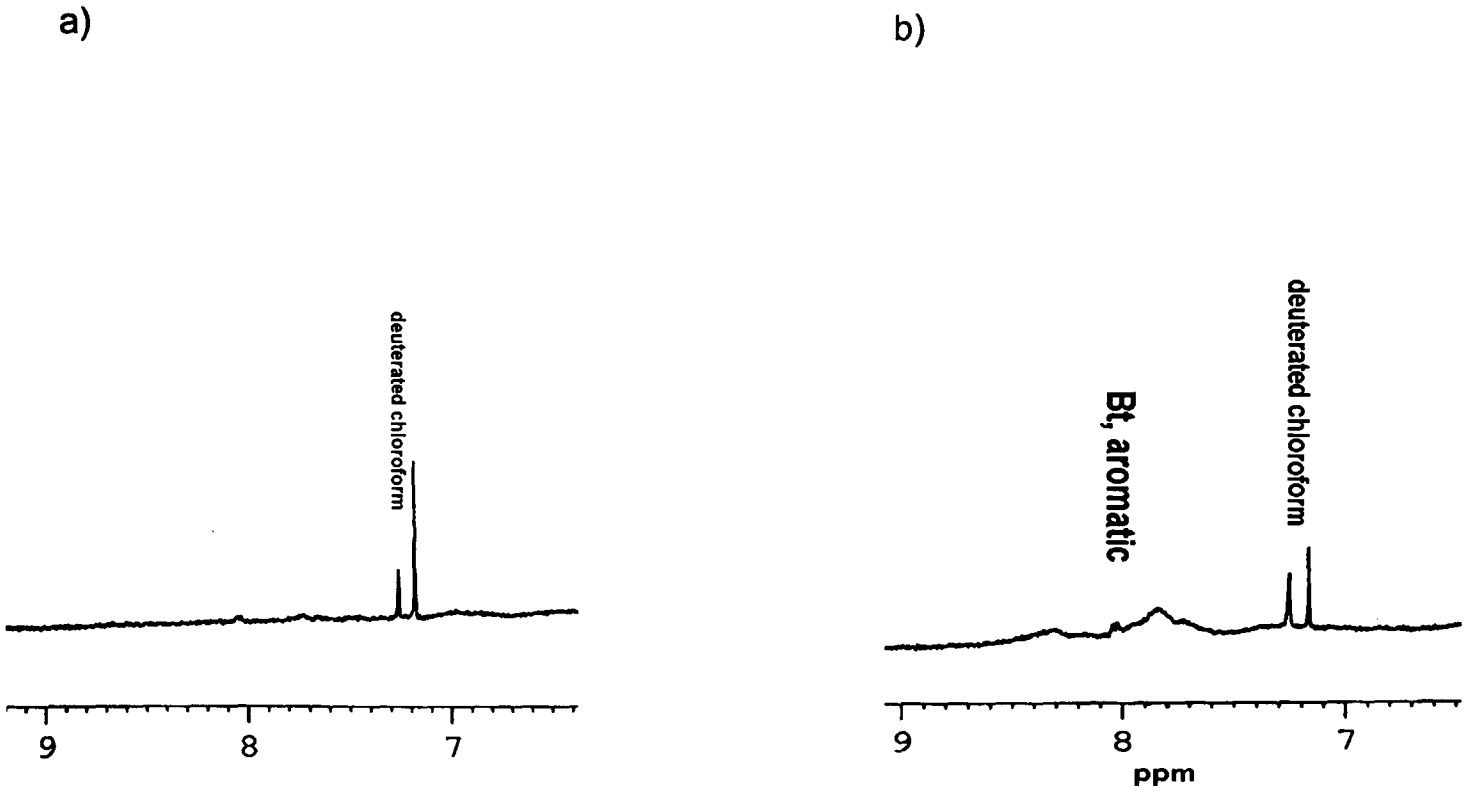


Figure 11: *High resolution MAS ^{1}H NMR spectrum of a) unfunctionalised $PEGA_{1900}$* ***216 and b) 220.***

A MAS ^{1}H NMR spectrum was not obtained for **217**, as no nanoprobe was available at the time of production of this intermediate. Additionally, MAS ^{1}H NMR was unobtainable for macroporous-polystyrene bound benzotriazole, **219**. Macroporous resin consists of a permanent network of pores which does not swell in any solvent. The poor swelling and rigidity of this resin means that only a small percentage of the attached substrate (*i.e.* exterior sites) is available to be detected by NMR which results in negligible signals in the MAS ^{1}H NMR spectrum.

In the case of aminomethyl-polystyrene and amino-methyl-polystyrene macroporous adducts, **218** and **219** respectively, gel phase carbon-NMR was unproductive. Similar problems in acquiring gel phase carbon-NMR were encountered by Schiemann and Showalter.[101] They reported that attempts to gather gel-phase ^{13}C NMR data on polymer supported benzotriazole **221** were unsuccessful, yet they were able to obtain spectra for the more flexible linkers **222** and **223** (Figure 12). Similarly, Katritzky *et. al.*[100] could not obtain a resolved gel phase ^{13}C spectrum for benzylic ether linked 1*H*-benzotriazole **182,** even after a lengthy acquisition (Scheme 50). It is thought that the lack of mobility in the polystyrene bound 1*H*-benzotriazole moiety of **218** and **219** inhibits the ability to obtain good quality gel

phase ^{13}C NMR. In the case of macroporous polystyrene adduct **219,** the mobility effect is magnified as macroporous resin is non-swelling in all common organic solvents. One drawback of gel-phase ^{13}C NMR on resin bound material is the low sensitivity which results from the small amount of compound attached to the resin. For this reason, gel-phase ^{13}C NMR was unattainable for low loading $PEGA_{1900}$ bound 1*H*-benzotriazole **220** (max; 0.2 mmol g^{-1}).

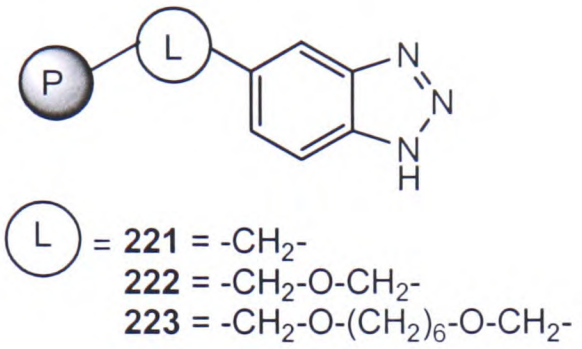


Figure 12: *Various linker lengths for polymer bound 1H-benzotriazole* ***221-223****.*

The lack of good analytical methods for resin bound samples has been a long-standing problem.[141] For example, conventional NMR techniques require samples to be relatively concentrated and homogeneous in order to achieve good spectral resolution. In order to try to overcome the difficulties associated with inhomogeneous samples, resin bound samples are pre-swollen in solvent. This technique is called 'gel-phase' NMR. Gel-phase proton NMR is limited in that poor resolution is obtained due to very broad linewidths (>100-300 Hz) which hamper any attempt to analyse resin bound samples. Gel phase ^{13}C NMR has inherent problems associated with insensitivity of the technique due to the low abundance of the ^{13}C nuclei; in addition, it also exhibits broad linewidths (*e.g.* 25-75 Hz). None the less, the wide range of chemical shifts associated with ^{13}C NMR (0-220 ppm) means that some structural information can usually be gained from the higher loading swelling resins (*e.g.* polystyrene) and the lower loading swelling/non-swelling resins (*e.g.* PEGA, Tentagel) are difficult to analyse by gel-phase ^{13}C NMR without incorporating ^{13}C labels.[142]

The advent of magic angle spinning (MAS) NMR has overcome some of the problems associated with obtaining ^{1}H and ^{13}C NMR spectra of heterogeneous samples. In theory, significant line narrowing (*e.g.* 4 Hz) can be achieved for resin

bound samples using MAS NMR since the effects of heterogeneous samples are lessened by spinning the sample at an angle to the applied magnetic field (54°). In practice, variable quality spectra are obtained depending on the resin and solvent used. Resins that offer the bound compounds the greatest mobility, such as PEG based and low cross-linked resins, produce the narrowest ^{1}H NMR linewidths.[143] In addition, the choice of solvent is crucial to the success of MAS spectra since the resin and bound compound is required to be swollen in the solvent.

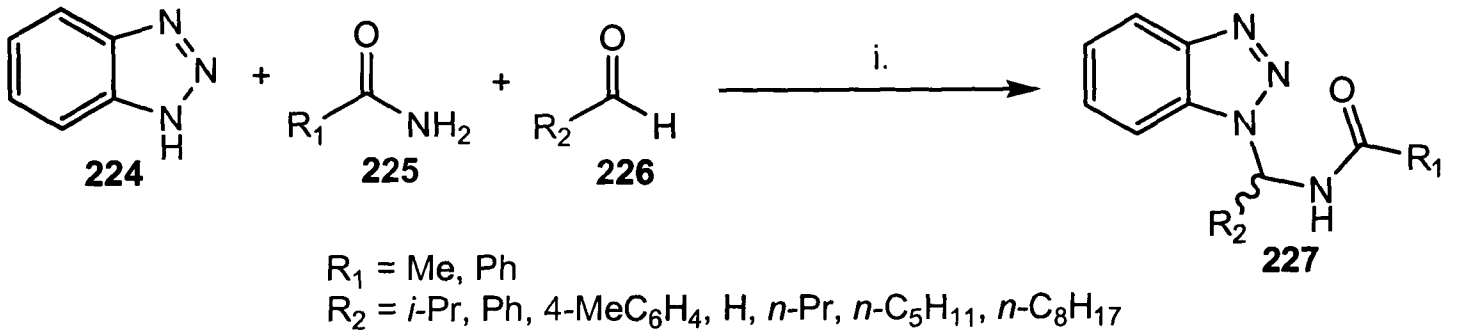
3.3 Summary and conclusions

Polymer bound 1*H*-benzotriazole adducts **217-220** were prepared using normal coupling procedures (*i.e.* diisopropylcarbodiimide, HOBt). The couplings were monitored by visual inspection (brown beads/gel indicated coupling) and the colourimetric Kaiser test. The presence of amide stretches in the FT-IR spectra of **217-219** indicated the formation of the amide bond; however, in the case of $PEGA_{1900}$ adduct, **220** the polymer backbone amide stretches overlapped with the new amide bond. Therefore, magic angle spinning ^{1}H NMR spectrum indicated the formation of **220**. Problems were encountered when attempting to aquire gel phase ^{13}C NMR of polystyrene resins **218-219** and gel phase carbon-NMR spectra were unobtainable due to the lack of mobility in these resins. The low loading of $PEGA_{1900}$ resin **220** hampered attempts to obtain a gel-phase carbon-NMR spectrum.

4 Results and discussion - Generation of aminal linkers

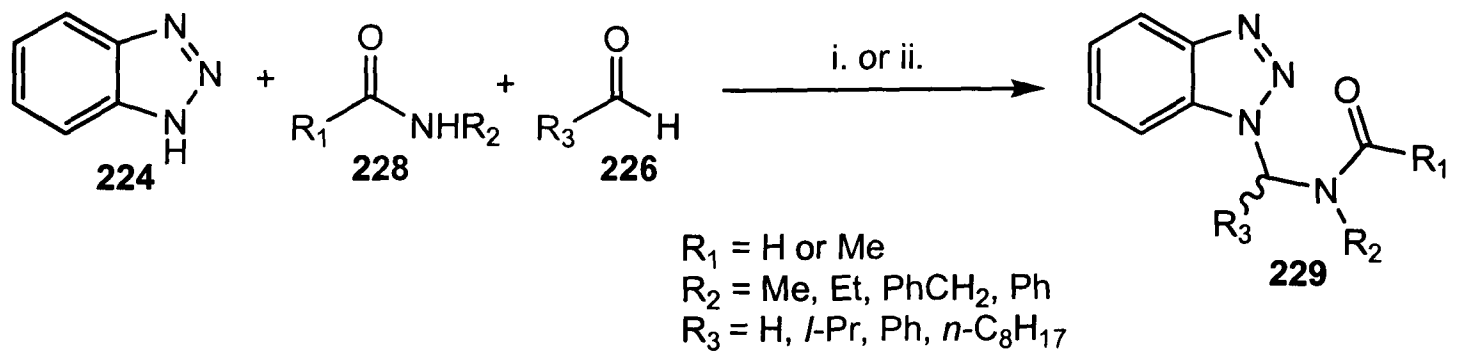
4.1 Reported syntheses of 1-(benzotriazol-1-yl)alkyl aminals

In 1988, Katritzky *et. al.*[81] described the formation of 1-(benzotriazol-1-yl)alkyl aminals **227** by condensation of an amide **225** with an aldehyde **226** and benzotriazole **224**. This was achieved by refluxing in dry toluene with azeotropic removal of water (Scheme 56), in yields ranging from 52-78%.



Scheme 56: *Reagents and conditions:* i. anhydrous toluene, reflux, Dean-Stark apparatus, 52-78%.

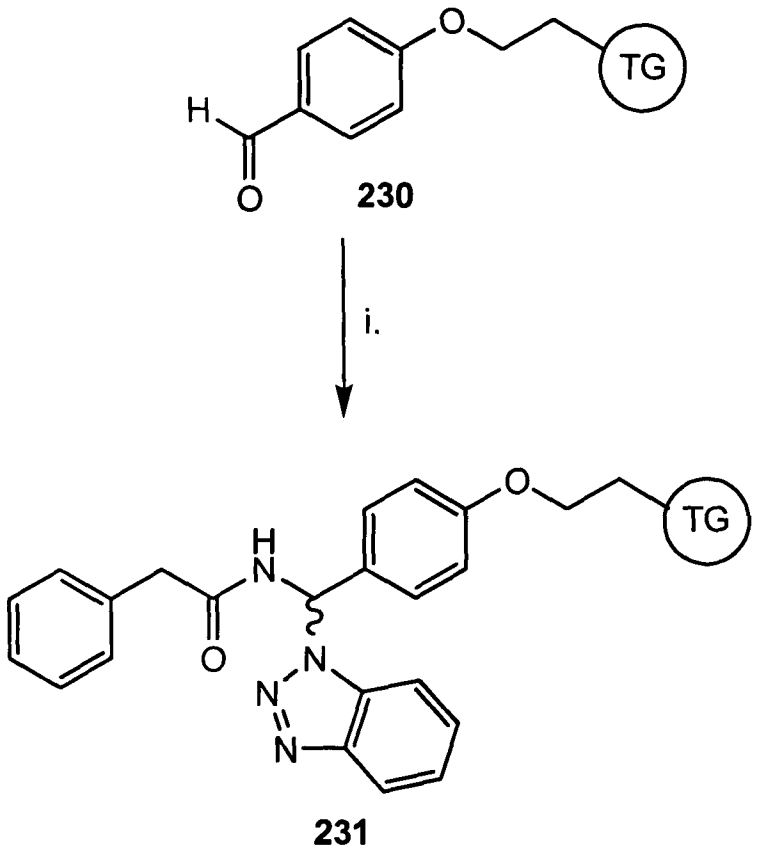
The preparation of condensation products from an amide **225**, aldehyde **226** and benzotriazole **224** was initially thought to be limited to primary amides **227**. However, in a later report by Katritzky *et. al.*[106], it was found that the use of catalytic amounts of *p*-toluene sulfonic acid, or carrying out the reaction in acetic acid facilitated the formation of secondary aminal benzotriazole adducts **229** (Scheme 57).



Scheme 57: *Reagents and conditions:* i. AcOH, reflux, Dean-Stark apparatus; ii. *p*-TsOH, toluene, Dean-Stark apparatus.

Previous work in our group[107], used the methodology described above (Scheme 57) to generate a novel linker **231** suitable for the attachment of alcohols to solid

support (Scheme 58). Moderate loading yields were reported (50%), which was probably due to the forcing conditions needed for removal of water *i.e.* azeotropic removal with Dean-Stark apparatus. Preliminary enzyme hydrolysis experiments using penicillin acylase met with limited success (10%). However, only TentaGel resin was tested and the enzymatic cleavage conditions were not optimized.



Scheme 58: *Reagents and conditions:* i. Phenylacetamide, benzotriazole, *p*-toluene sulfonic acid, reflux under Dean-Stark Conditions, 18 h.

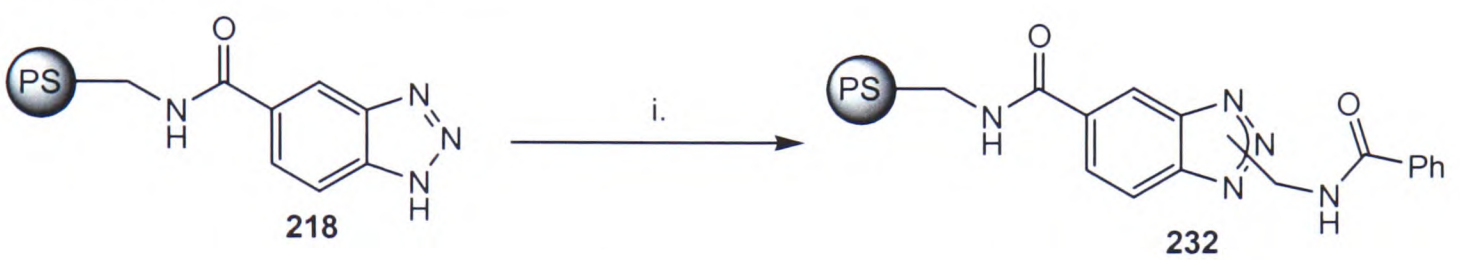
Initial studies concentrated on the syntheses and enzyme hydrolyses of 1-(benzotriazolyl)alkyl aminal linkers since the methodology for synthesis of these linkers was established,[107] albeit in a inverted manner (Scheme 58).

4.2 Synthesis of polystyrene-bound 1-(benzotriazol-1-yl)alkyl aminals

The synthetic schemes outlined above (Scheme 56/Scheme 57) allow the generation of a range of 1-(benzotriazole-1-yl)alkyl aminals **227/229** simply by manipulating the aldehyde **226** or amide **225/228** used. In view of the work by Flitsch *et. al.*[50], it seemed reasonable that this chemistry could be transferred directly to solid phase to produce polymer bound 1-(benzotriazole-1-yl)alkyl aminals. A range of these aminals were prepared on a benzotriazole solid support and subjected

to various investigative tests. In addition, the resin bound aminals were incubated with penicillin acylase to determine if the enzyme can cleave the aldehyde from solid support.

Polystyrene-bound 1-(benzotriazole-1-yl)formyl aminal **232**, was produced from the condensation of benzotriazole resin **218**, benzamide and formaldehyde to give **232** in moderate loading (58%) (Scheme 59). The azeotropic removal of water was achieved through the use of Dean-Stark apparatus. Formation of linker **232** was confirmed by a signal at ~5.4 ppm in the ^{1}H MAS NMR spectrum corresponding to the methylene aminal protons.



Scheme 59: *Reagents and conditions:* i. formaldehyde (37% in H_2O/MeOH), benzamide, *p*-toluene sulfonic acid, anhydrous toluene, 48 h, reflux, Dean-Stark apparatus, 58% loading.

The cleavage of the linker **232** was observed using two acidic cleavage methods; trifluoroacetic acid/dichloromethane/water (9:10:1) and 95% trifluoroacetic acid in water. This was optimised by measuring the amount of benzamide released, at 15 minute intervals over 4.75 hours. Experimental conditions are given in Section 8.6.11.1.

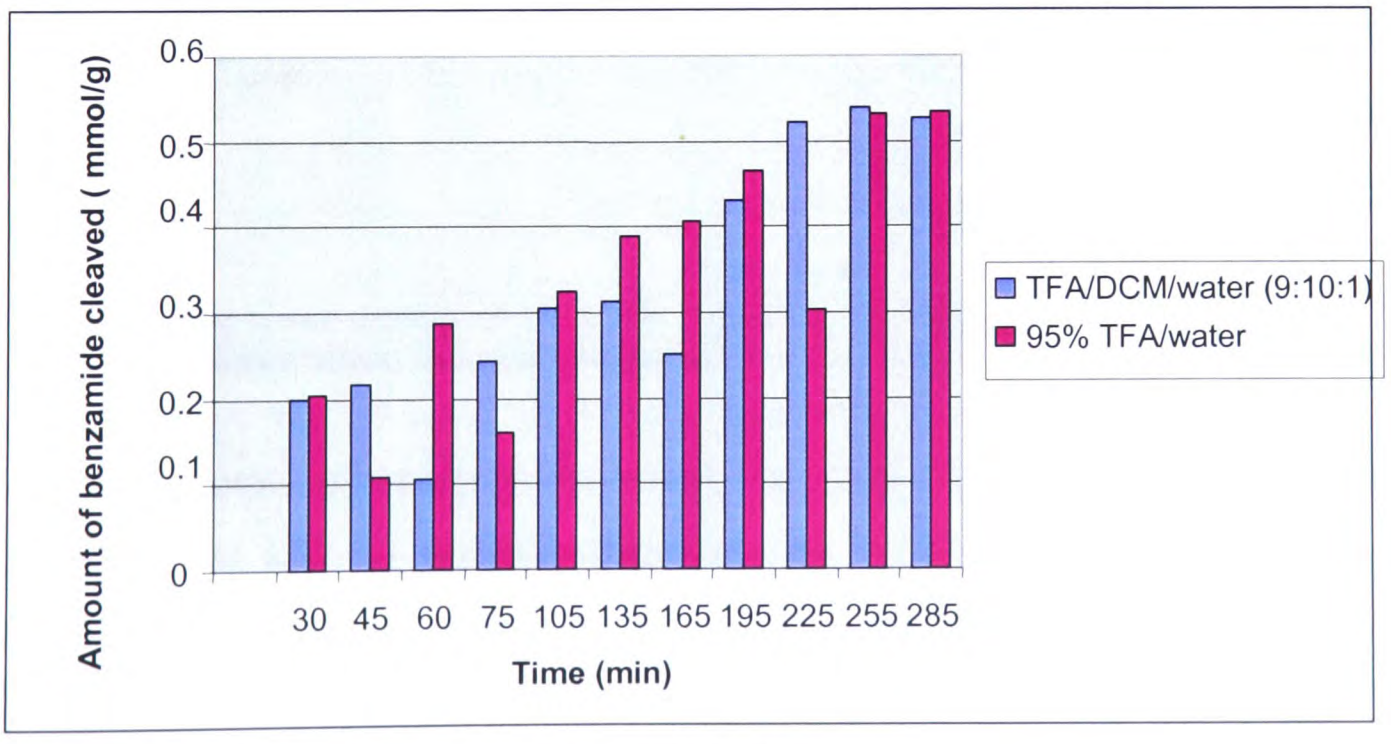
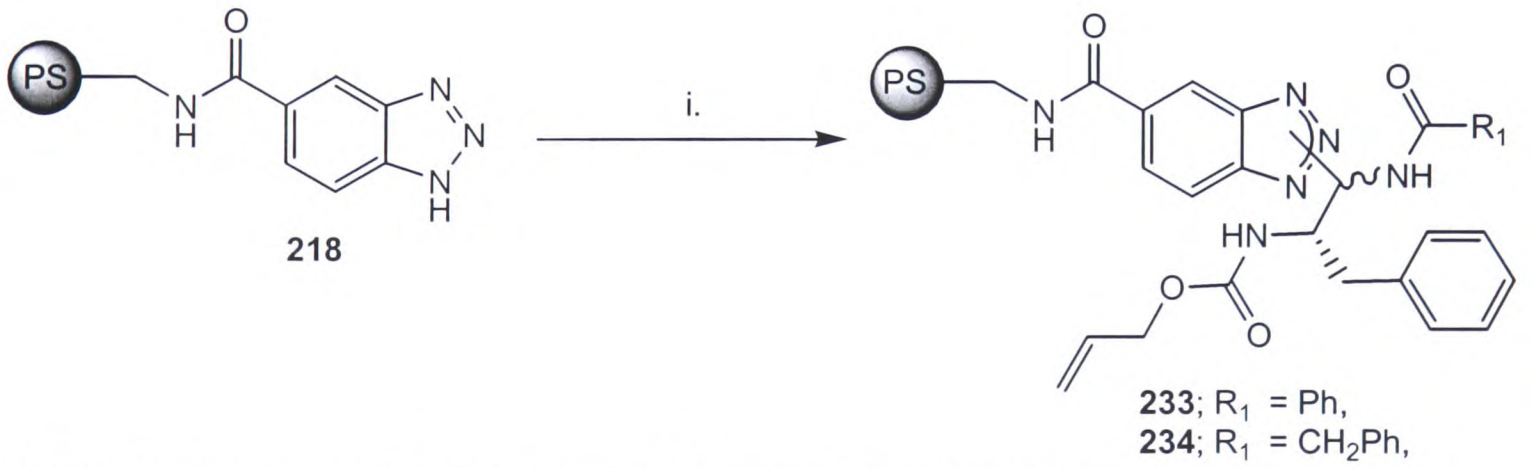


Figure 13: *Cleavage optimization of aminal linker* ***232****.*

From Figure 13, it can be seen that both cleavage methods, TFA/DCM/H_2O and 95% TFA/H_2O, follow a similar trend. In both cases, similar cleavage rates are observed and quantitative cleavage is obtained in 255 minutes (4.25 hours). This is in spite of the fact that, the hydrophobic polystyrene resin swells better in TFA/DCM/H_2O (9:10:1) than 95% TFA/H_2O. The anomalous results (*e.g.* at 45 min for 95% TFA cleavage) are possibly caused by inhomogeneous loading on the polymer matrix. Each measurement was made on different samples of resin **232**. On-line monitoring of acidic cleavage using a Gilson SK233 robot and Water HPLC system produced unreliable results due to concentration gradients in the reaction mixture. This was caused by insufficient mixing inside the Irori™ can. Irori™ cans are small porous vessels which are used in combinatorial chemistry for the containment of resins. In light of the increased swelling ability, the TFA/DCM/H_2O (9:10:1) cleavage mixture was chosen for future aminal linker cleavage.

The synthesis of polystyrene bound aminal linkers **233** and **234**, was carried out; with a view to, demonstrating the ability to synthesise peptide aldehydes on solid support.

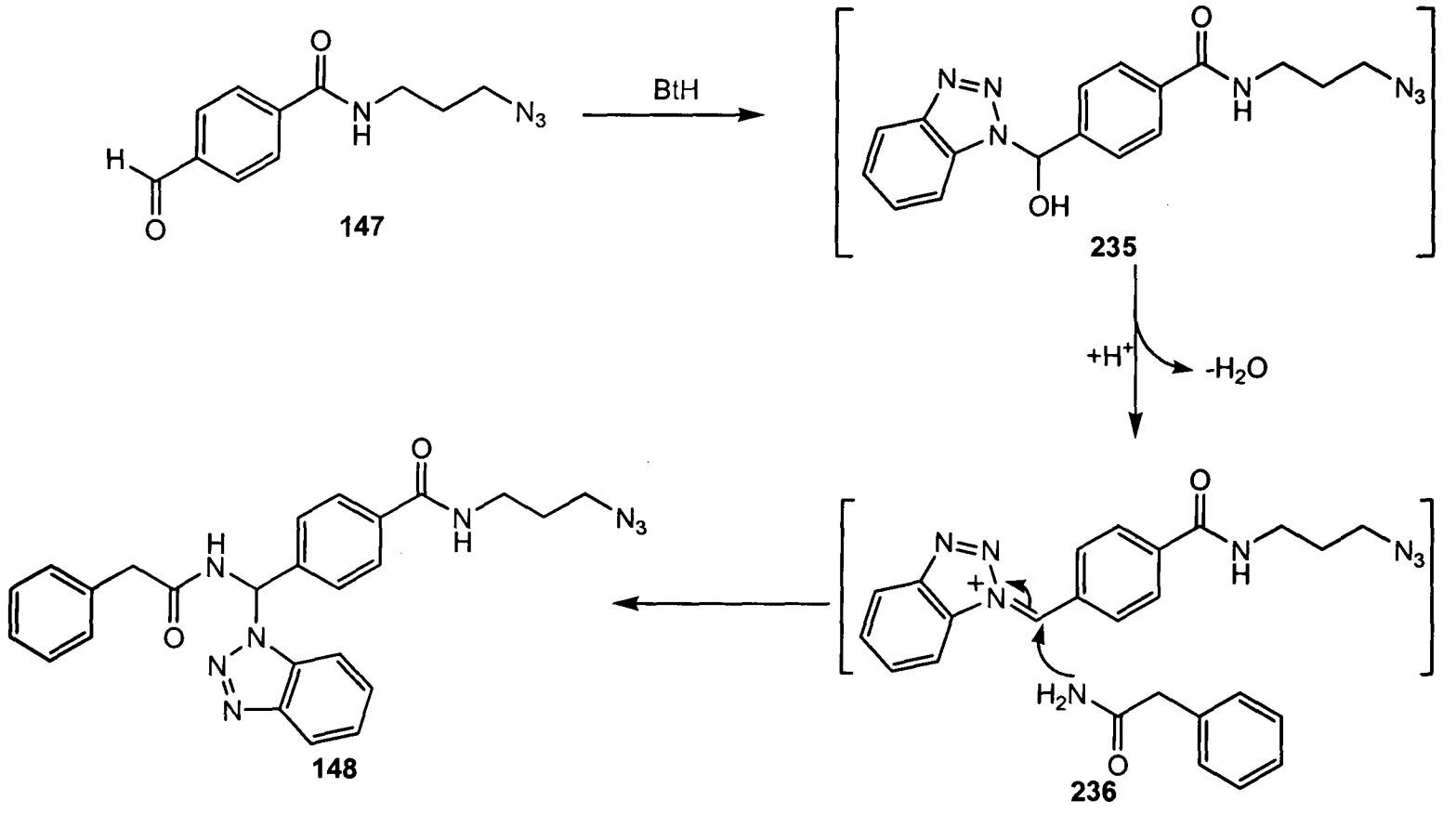


Scheme 60: *Reagents and conditions:* i. (*S*)-**171**, benzamide or phenylacetamide, *p*-toluene sulfonic acid, anhydrous toluene, reflux, Dean-Stark apparatus.

Condensation of polystyrene-bound benzotriazole **218**, benzamide or phenylacetamide and (*S*)-allyloxycarbonyl-phenylalaninal **171** in the presence of catalytic *p*-toluenesulfonic acid under Dean-Stark conditions afforded the coupled linkers **233** and **234**. During refluxing, a white solid precipitated overnight which was difficult to remove by washing due to the insolubility in most organic and

aqueous solvents; DMF, DCM, DMSO, H_2O, $MeOH/H_2O$, $(CH_3)_2CO$, Et_2O, EtOAc, CH_3CN. Only prolonged washings with copious amounts of solvents; DMF, MeOH, DCM (~200 mL), in combination with sonication, removed the unwanted solid. Since, this was not observed when formaldehyde was employed as the aldehyde component in **232**, it was reasonable to assume that the solid was associated with allyloxycarbonyl-phenylalaninal **171**. Initially, **171** was soluble in toluene; however only after reflux overnight in the presence of the rest of the reagents, did the insoluble white solid become evident. It was thought that the presence of the *p*-toluene sulfonic acid caused the allyloxycarbonyl-phenylalaninal **171** to polymerise. Nonetheless, FT-IR confirmed the presence of the urethane carbonyl (~1700 cm^{-1}) in both cases **233** and **234**. Additionally, MAS ^1H NMR of **233** showed the Alloc protecting group at 4.30-4.67 ppm and 4.90-5.24 ppm.

In 2000, the mechanism for the formation of **148** was proposed by Rice[82] and Burgess[108] (Scheme 61). The first step involves nucleophilic attack of the benzotriazole nitrogen on the aldehyde carbon **147**, followed by acid catalysed loss of water. Benzotriazole possesses both electron-donor and -acceptor properties and in this case acts both as a weak acid ($pK_a = 8.2$) and as a nucleophile.



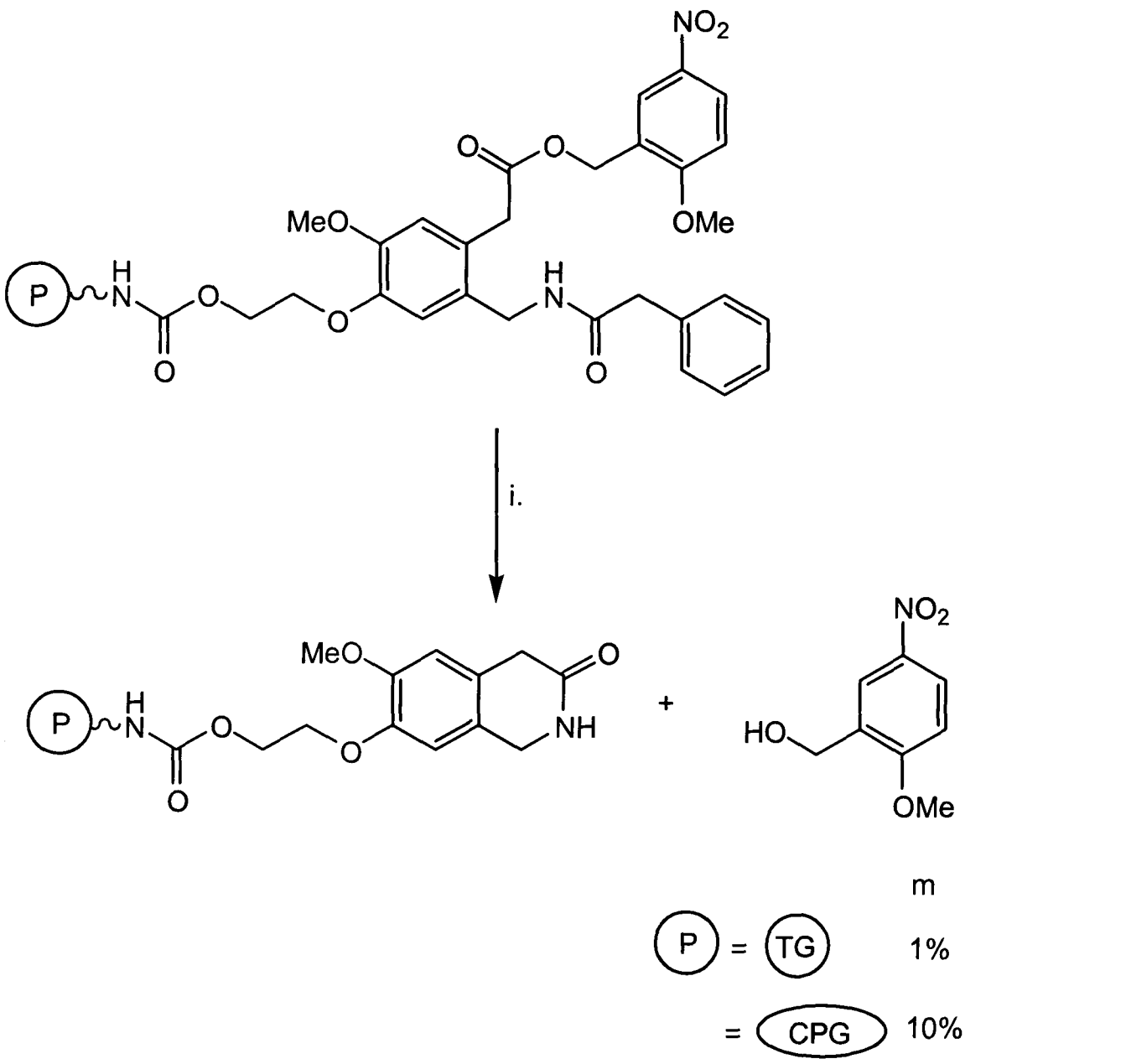
Scheme 61*: Proposed mechanism for the formation of **148**.*

The cleavage of **233** in TFA/DCM/H_2O (9:10:1), followed by chiral HPLC (Section 8.2.9), was performed to ascertain whether (*S*)-allyloxycarbonyl-phenylalaninal **171** was generated from the solid support with retention of the chiral purity. The chiral HPLC of the cleaved and reduced product (*i.e.* allyloxycarbonyl-phenylalaninol **174**) was compared to a sample of chemically prepared racemic allyloxycarbonyl-phenylalaninol **175** to allow for the accurate determination of chiral purity. Initially, a small sample of resin **233** (30 mg) was cleaved; however, it was found that the resolution of the chiral HPLC method was not sufficient to allow for an accurate determination of enantiomeric excess. Therefore, a large sample of resin **233** (238 mg) was treated with TFA/DCM/H_2O for 4 h. The residue obtained was reduced with sodium borohydride and analysed by chiral HPLC. The chiral HPLC trace showed one peak at a retention time of 30.5 min corresponding to (*S*)-allyloxycarbonyl-phenylalaninol **174** with 97% enantiomeric excess. This demonstrates the potential for cleaving amino aldehydes **171** from the aminal linker **233** with complete retention of chiral purity, using an acidic cleavage method (TFA/DCM/H_2O 9:10:1).

4.3 Enzymatic hydrolysis of polymer-bound 1-(benzotriazolyl)propyl aminals.

Polystyrene-based supports are used in a variety of classical solid phase organic reactions; however, due to the hydrophobic nature of this polymer, polar reagents fail to enter the matrix. Polystyrene can be modified by grafting poly(ethylene glycol) to the hydrophobic core to produce a polymer that swells in both non-polar and polar solvents. Among these PEG-grafted polystyrene supports, TentaGel has been used extensively in solid phase synthesis because of the mechanical stability of the beads and the good swelling properties in organic and aqueous media. However, TentaGel is based on the short cross linking agent divinylbenzene at a level of 1-2%, which prevents access to most enzymes[20]. Waldmann *et. al.*[43] showed that only the outer surface of the TentaGel bead is accessible to the biocatalyst (penicillin G acylase 80 kDa) (Scheme 62). Recently, Bradley *et. al.*[14] have shown that even small enzymes (12 kDa) are not accessible to TentaGel beads. In contrast, a support based on

poly(ethylene glycol) as the major component of the resin, is more compatible with aqueous media[36] and should allow access to enzymes. PEGA consists of a co-polymer of *bis*-2-acrylamidoprop-1-yl polyethylene glycol and the rapid diffusion of even large biomolecules in the interior of the PEGA polymer network, has recently been confirmed by confocal raman microscopy.[14]



Scheme 62: *Reagents and conditions:* i. penicillin G acylase suspension, 10% methanol, 37 °C, 10% cleavage.

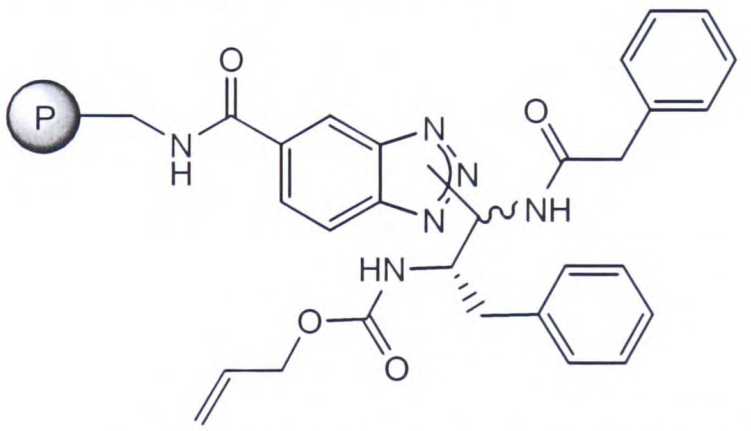
The problem of solvent compatibility is significantly reduced with the advent of rigid macroporous supports. Access of liquid reagents to the reaction sites occurs by rapid diffusion through the rigid, open pore structure rather than through a swollen gel phase. Additionally, accessibility of reaction sites is independent of solvent type. The macroporous polystyrene-*co*-divinylbenzene (MP) resins were invented in the 1950's[109] and since then, have been further developed to provide MP resins with larger pore sizes (>1000 Å), suitable for the diffusion of macromolecules. Another macroporous resin available is controlled pore glass (CPG). As mentioned already

(Section 1.2.5), it seems that a large pore size of CPG (>100 nm) is crucial to the success of biotransfomations on solid support.[14] Waldmann *et. al.*[44] described the use of CPG with a pore size of 50 nm in combination with a large enzyme (80 kDa), but only minor cleavage (10%) of a model compound from this support was detected (Scheme 62).

Two enzyme labile aminal linkers **237** and **238** were prepared and their compatibility with enzymatic hydrolysis tested.

4.4 Synthesis of enzyme-labile polymer bound 1-(benzotriazole-1-yl)propyl aminals.

Macroporous polystyrene and TentaGel bound 1-(benzotriazole-1-yl)alkyl aminals **237** and **238** were obtained using the procedure described for polystyrene resin **233** and **234** (*i.e.* Scheme 60) (Figure 14).



237; P = macroporous aminomethyl polystyrene
238; P = TentaGel

Figure 14: *Enzyme labile aminal linkers **237** and **238**.*

The FT-IR of macroporous resin, **237** was inconclusive since the urethane carbonyl stretch (~1720 cm^{-1}) was masked by anomalous polymer backbone carbonyl stretches. In addition, Gel phase ^{13}C and MAS ^{1}H NMRs were unattainable due to the non-swelling character of the macroporous support. In this case, the linker **237** was carried through to the next step. In the case of TentaGel resin **238**, the presence of a urethane carbonyl stretch at 1717 cm^{-1} confirmed the success of the coupling (Figure 15). No MAS probe was available at this time.

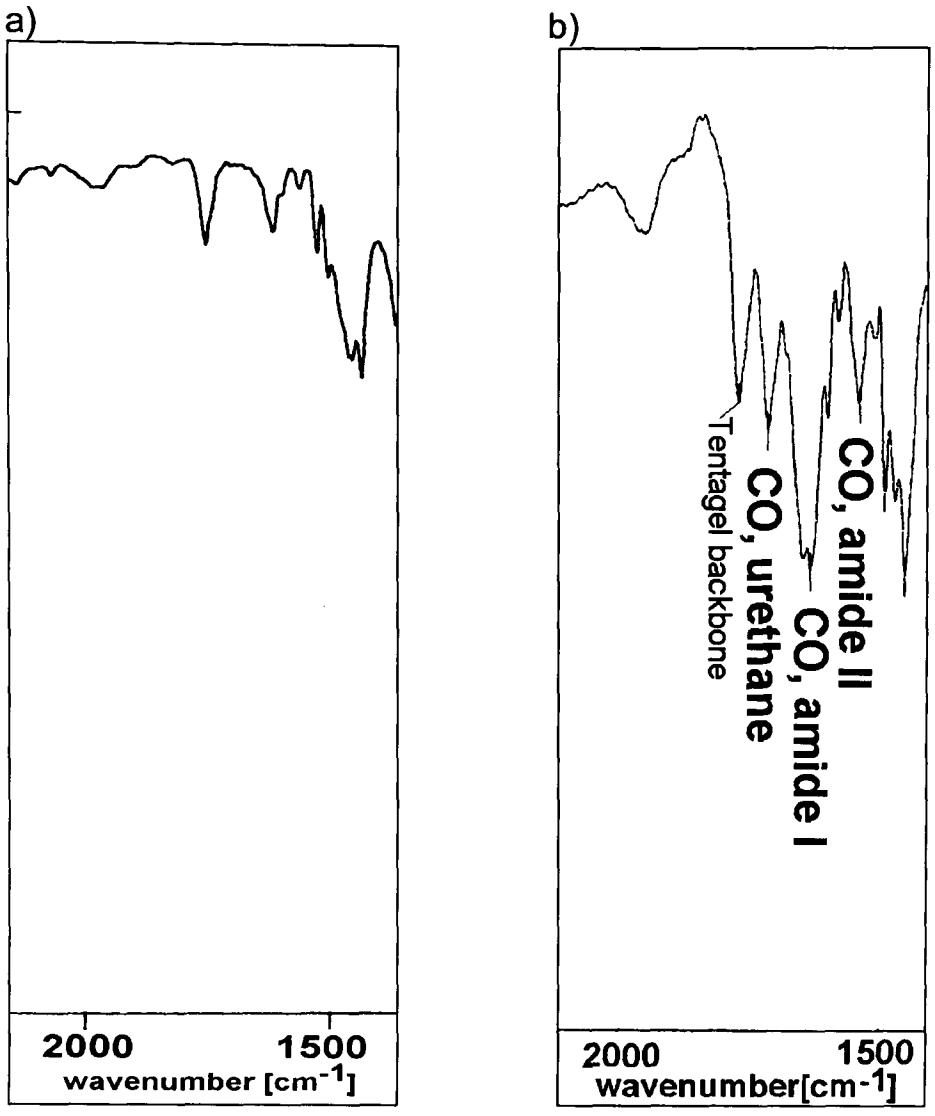


Figure 15: *FT-IR spectrum of a) unfunctionalised amino tentagel (KBr disc) and b)* ***238*** *(KBr disc).*

4.5 Enzyme hydrolysis studies

The compatibility of resins **237** and **238** towards penicillin acylase mediated hydrolysis was assessed. Enzyme hydrolysis experiments were performed by Dr Rein Ulijn at Edinburgh University. The resins **237** and **238** were treated with penicillin acylase in phosphate buffer pH 7.4 for 16 hours and the amount of phenylacetic acid released was analysed by reverse phase HPLC (Section 8.2.9). As expected, the hydrolysis of TentaGel bound linker **238** with penicillin acylase only resulted in surface sites being cleaved (*i.e.* <12% cleavage). This is in agreement with studies carried out by Barany *et. al.*,[24] who reported that only the surface sites on TentaGel resin were accessible to biomolecules. It was expected that enzyme hydrolysis on macroporous polystyrene linker **237** would be more successful due to the large rigid pores. However, penicillin acylase mediated hydrolysis on macroporous resin **237** was very disappointing and the maximum cleavage yield was <5%. This result suggests that the hydrophobic aromatic backbone of macroporous

polystyrene resin repels the hydrophilic enzyme; therefore, inhibiting access of the enzyme to the interior of the macroporous resin.

4.6 Summary and conclusions

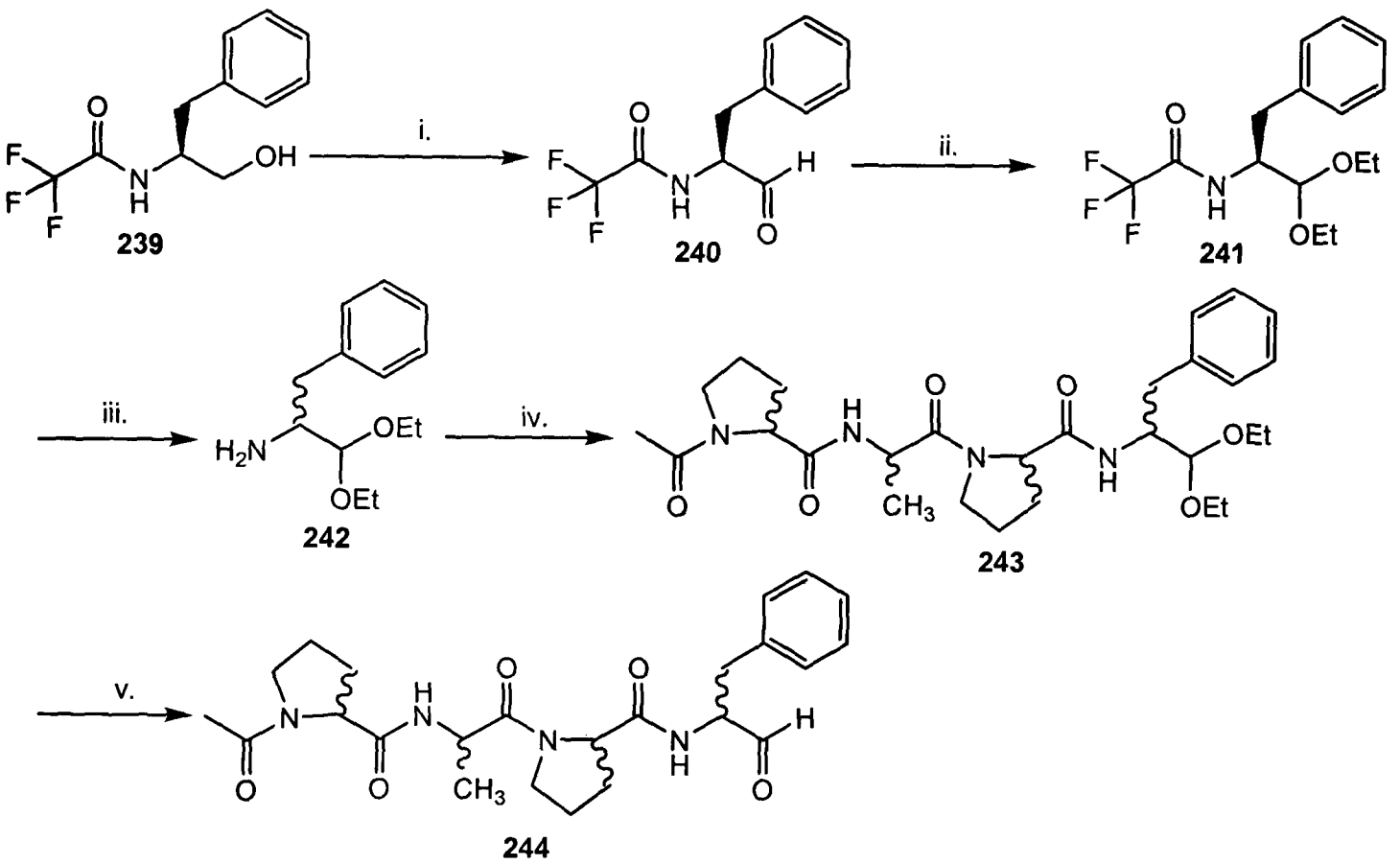
The synthesis of various aminal linkers **232-234** and **237-238** has been demonstrated. Cleavage optimization of polystyrene linker **232** was performed. It was discovered that the best cleavage conditions were TFA/DCM/H_2O (9:10:1) for 4.25 hours. The potential for cleaving amino aldehydes from these aminal linkers was demonstrated by acidic cleavage of **233** which afforded (*S*)-allyloxycarbonyl-phenylalaninal **171** in 97% enantiomeric excess.

Enzymatic hydrolysis of macroporous polystyrene and TentaGel aminal linkers, **237** and **238** was carried out. Penicillin acylase mediated hydrolysis of TentaGel resin **238** only resulted in surface sites being cleaved (<12% cleavage). Whereas, <5% hydrolysis was observed for macroporous aminal linker **237**.

5 Results and discussion - Solid-phase peptide aldehyde synthesis on aminal linker.

5.1 Reported synthesis of tetrapeptide.

It was thought that, the synthesis of a simple tetrapeptide would be a straightforward and rapid way of demonstrating the scope of the linkers, **233** and **234**. Thompson *et. al.*[55] reported the solution phase synthesis of the tetrapeptide aldehyde, acetylprolylalanylprolyl-phenylalaninal **244** *via* diethyl acetyl masked phenylalaninal **242** (Scheme 63).



Scheme 63: *Reagents and conditions:* i. 1-ethyl(3,3-dimethylaminopropyl)carbodiimide hydrochloride, DMSO, 2 M solution of anhydrous phosphoric acid in DMSO, 3 h; ii. *p*-toluene sulfonic acid, triethylorthoformate, ethanol, 30 min; iii. Sodium hydroxide (1 M), methanol, 45 min, H_2O, r.t.; iv. acetylprolylalanylproline, *N*-methylmorpholine, acetonitrile, -20 °C, isobutylchloroformate, then r.t for 4 h; v. Dowex AG50W resin (H^+ form), r.t., 2.5 h.

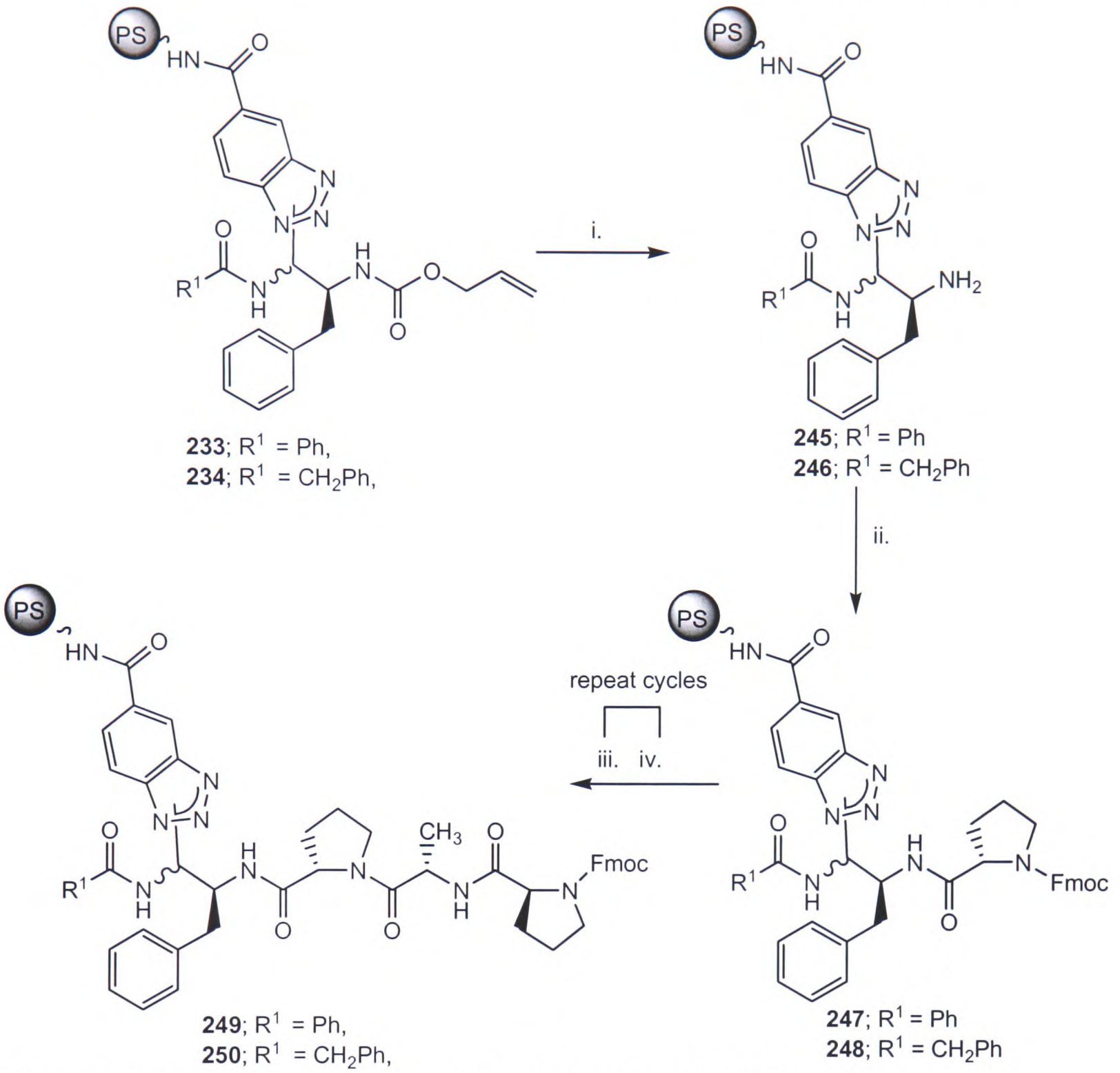
The peptide aldehyde, prolylalanylprolylphenylalaninal **244** was chosen for solid phase synthesis using the aminal linkers **233** and **234**. Due to the relatively poor enzymatic cleavage yields for **237** and **238**, it was decided to target chemical methods for the cleavage of the peptide aldehyde from solid support; for this reason,

aminomethylated polystyrene resins, **233** and **234,** were chosen for the synthesis of the peptide aldehyde.

5.2 Solid-phase peptide aldehyde synthesis on aminal linker.

Deprotection of allyloxycarbonyl from **233** and **234** was achieved with borane-dimethylamine complex and soluble palladium catalyst to afford **245** and **246** (Scheme 64). This combination allows the rapid deprotection (10 min) of allyl carbamates under nearly neutral conditions and without any side-formation of allylamine. The reaction was monitored by the Kaiser test (Section 8.2.5.1) and the FT-IR spectrum of resin bound free amine **245,** showed complete disappearance of the carbonyl urethane stretch at ~1700 cm^{-1}.

Coupling of Fmoc-proline to the free amine resins **245** and **246** was carried out using the common coupling reagents, HBTU and HOBt in the presence of DIPEA as base. Complete coupling was achieved when a negative Kaiser test (Section 8.2.5.1) was observed after 4 hours, for **247** and **248**. Fmoc deprotection was accomplished with 20% piperidine in DMF. The amino acid coupling procedure described above was repeated with Fmoc-alanine, followed by Fmoc deprotection. The final amino acid, Fmoc-proline was coupled using the same procedure.



233; R^1 = Ph,
234; R^1 = CH_2Ph,

245; R^1 = Ph
246; R^1 = CH_2Ph

249; R^1 = Ph,
250; R^1 = CH_2Ph,

247; R^1 = Ph
248; R^1 = CH_2Ph

Scheme 64: *Reagents and conditions:* i. borane-dimethylamine complex, $Pd(PPh_3)_4$, anhydrous DCM, 10 min, r.t; ii. Fmoc-Pro-OH, DMF, DIPEA, 0.45M HBTU/HOBt solution in DMF, 4 h, r.t.; iii. 20% piperidine in DMF, 20 min; iv. Fmoc-Ala-OH, DMF, DIPEA, 0.45 M HBTU/HOBt solution in DMF, 4 h, r.t.; v. 20% piperidine in DMF, 20 min; vi. Fmoc-AA-OH, DMF, DIPEA, 0.45 M HBTU/HOBt solution in DMF, 4 h, r.t.

Loading of the tetrapeptide aldehydes **249** and **250** was calculated at 0.16 (27%) and 0.28 mmol g^{-1} (48%) respectively using Fmoc analysis (Section 8.2.6.1). These loading yields were unexpected. The loading of benzamide linker **249** was expected to be higher than the phenylacetamide linker **250**. This may be explained by the differences in nucleophilicities of the amides. In the construction of linkers, **233** and **234**), nucleophilic amide reacts with benzotriazole imine **236** (Scheme 61). In the case of phenylacetamide (Route a; Figure 16), the lone pair of electrons on the amide

nitrogen can be delocalized onto the carbonyl to decrease the nucleophilicity of the nitrogen; however, in benzamide (Route b; Figure 16), there is less delocalization of the amide nitrogen electrons due to competing delocalization of the aromatic electrons onto the carbonyl. This renders the amide nitrogen on benzamide more nucleophilic than the phenylacetamide nitrogen. These loading yields suggest that steric hindrance is more important than electronic effects since the aryl ring is closer to the reactive amine in benzamide than phenylacetamide. Therefore, the aryl ring on benzamide hinders nucleophilic attack on benzotriazole imine **236**.

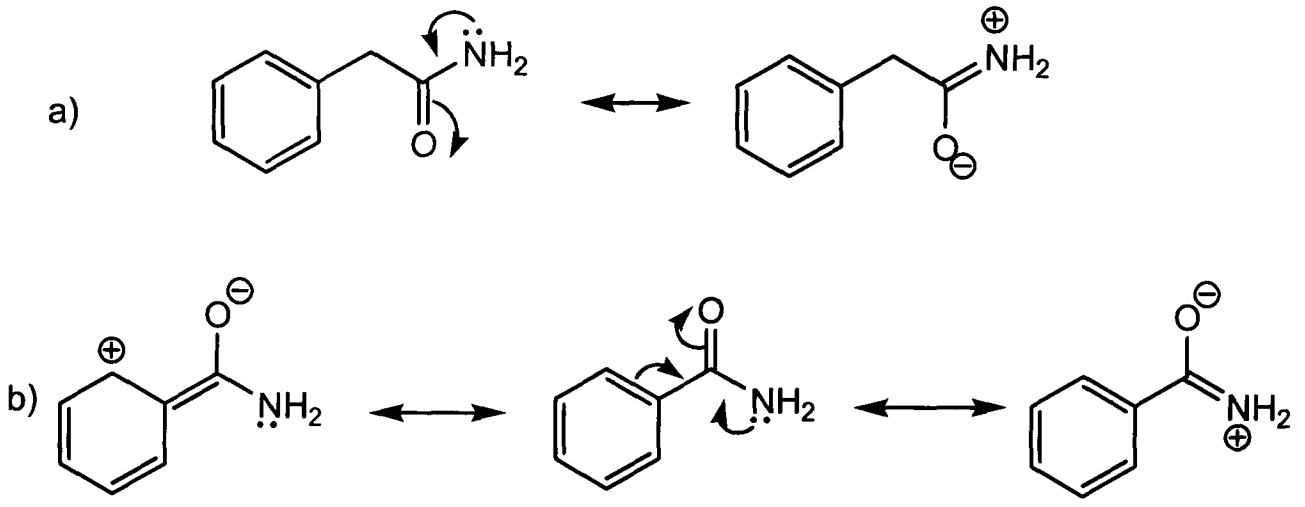


Figure 16: *Delocalization of electrons onto carbonyl carbon for a) phenylacetamide and b) benzamide.*

At the time of production of these resins **249** and **250**, the use of MAS ^{1}H NMR to analyse these resins was impossible due to the lack of a MAS probe within the department. Nonetheless, MAS ^{1}H NMR of **249** and **250** was carried out at a much later date. It should be noted that these amide linkers decompose slowly over about 12 months even at 4 °C. The spectrum of both, showed mainly broad signals which were too weak to assign confidently. Many of the proton signals were masked by the methine, methylene and aromatic signals from the polystyrene backbone. However, in both cases, two benzotriazole aromatic signals were evident at ~7.6 - 7.8 ppm, as was the methine aminal proton at ~6 ppm (Figure 17).

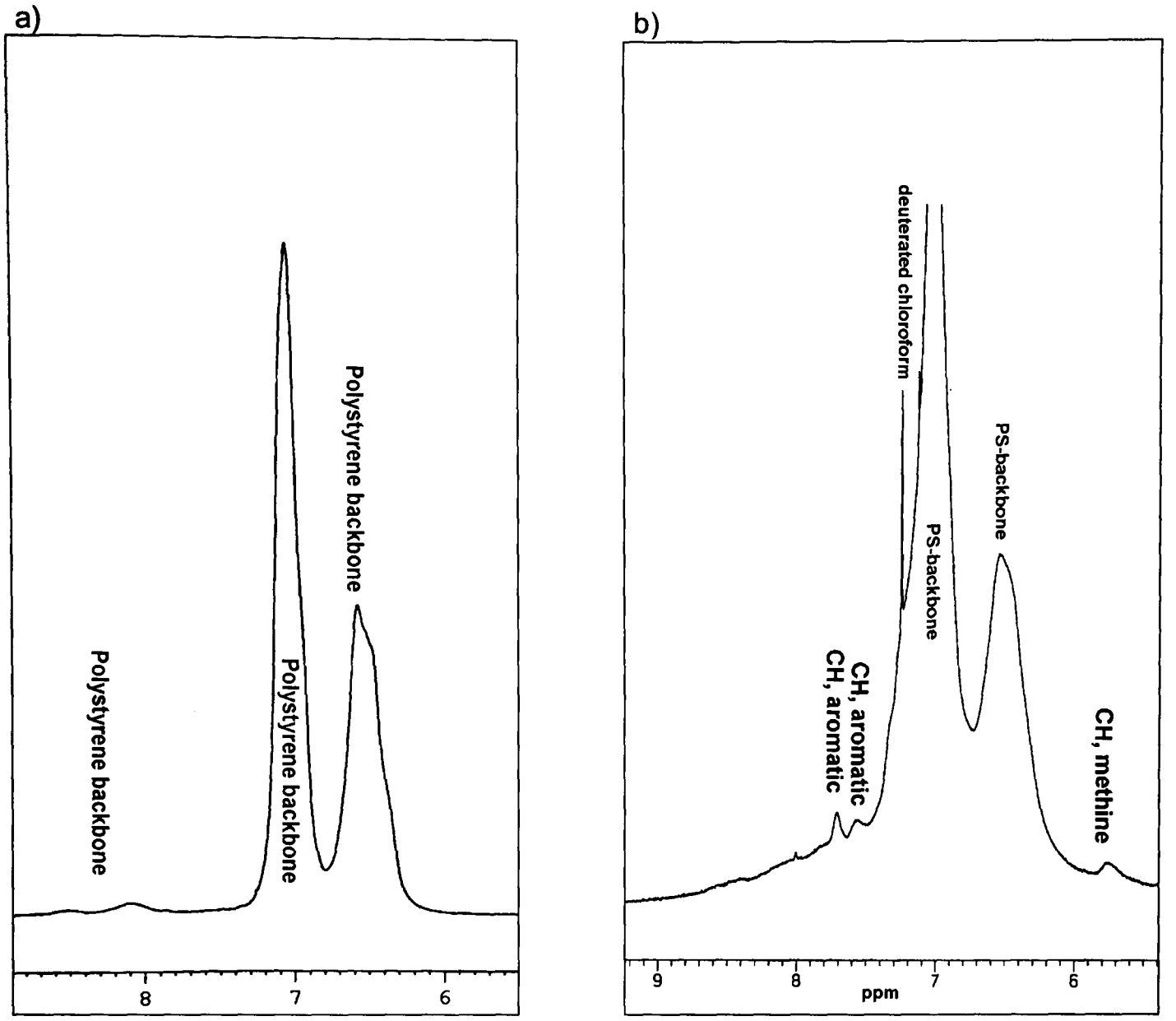


Figure 17: *MAS ^1H NMR spectrum of a) aminomethyl polystyrene **214** and b) **249**.*

Cleavage of both linkers **249** and **250** was carried out using TFA/DCM/H_2O (9:10:1) (Section 8.2.7.1), followed by LC-MS analysis (Section 8.2.8). In the case of **250,** the mass spectrometry clearly shows the desired MH^+ ion at 637 *m/z* at a retention time of 26 min (Figure 18). Further treatment with acid resulted in no further cleavage of peptide aldehyde indicating that cleavage was quantitative. Insufficient material was obtained for analysis by ^1H NMR, even at 600 MHz. Only broad unresolved peaks were obtained probably due to the nature of the peptide and insufficient material. Attempts were made to purify the peptide aldehyde using an automated mass directed fraction collector and Micromass ZMD mass spectrometer. This was unsuccessful due to insufficient material and problems with insensitivity of method since threshold settings for collection of the desired mass ion could not be lowered sufficiently.

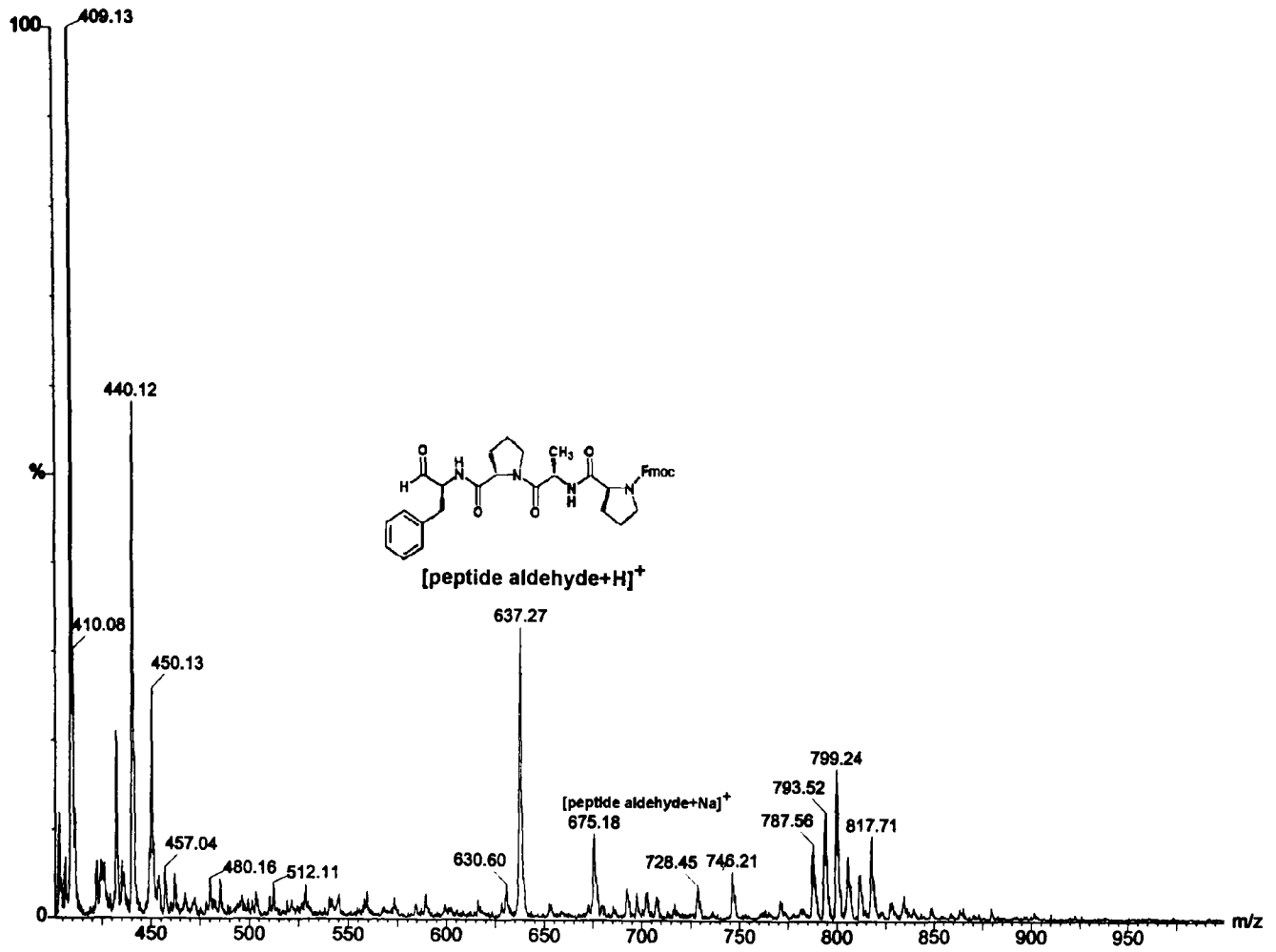


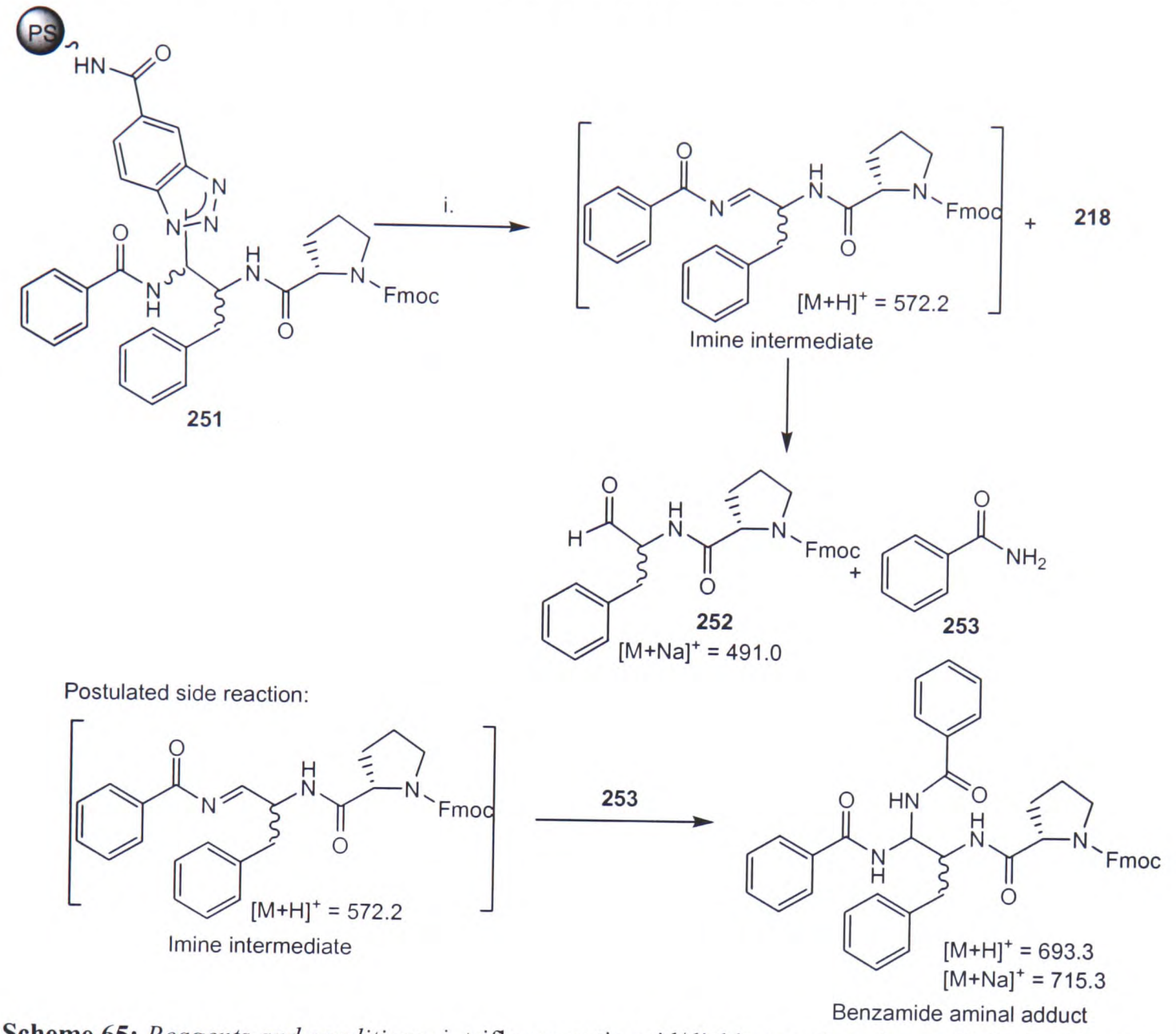
Figure 18: *ES-MS of **250** cleavage mixture.*

Unfortunately, cleavage of **249** (Scheme 64), resulted in no peptide aldehyde being detected by electrospray mass spectrometry. This suggested that one of the amide couplings was not successful since Fmoc was clearly present from the UV trace after piperidine cleavage; although, there were no MH^+ ions in the mass spectrum of **249** corresponding to depletions in the peptide backbone.

At this point, it was thought that the synthesis of a dipeptide aldehyde would simplify the analysis. Hence, dipeptide aldehyde linker **251** was synthesized using the same coupling procedures as used for the tetrapeptide aldehyde (Scheme 64). In this case, racemic allyloxycarbonyl-phenylalaninal **173** was used.

Acidic cleavage of **251** (Scheme 65), followed by purification using semi-preparative TLC ($R_f = 0.33$) and electrospray mass spectrometry (Figure 19) revealed the presence of the desired $[M+Na]^+$ ion at 491 *m/z* and another impurity which has been postulated to be the imine intermediate, $[M+H]^+$ and $[M+CH_3CN]^+$ ions at 572 and 612 *m/z* respectively. This imine intermediate can undergo nucleophilic attack

by liberated benzamide **253** to generate the benzamide aminal adduct, $[M+H]^+$ and $[M+Na]^+$ ions at 693 and 715 *m/z* respectively (Scheme 65 and Figure 19).



Scheme 65: *Reagents and conditions:* i. trifluoroacetic acid/dichloromethane/water (9:10:1), 4 h, r.t.

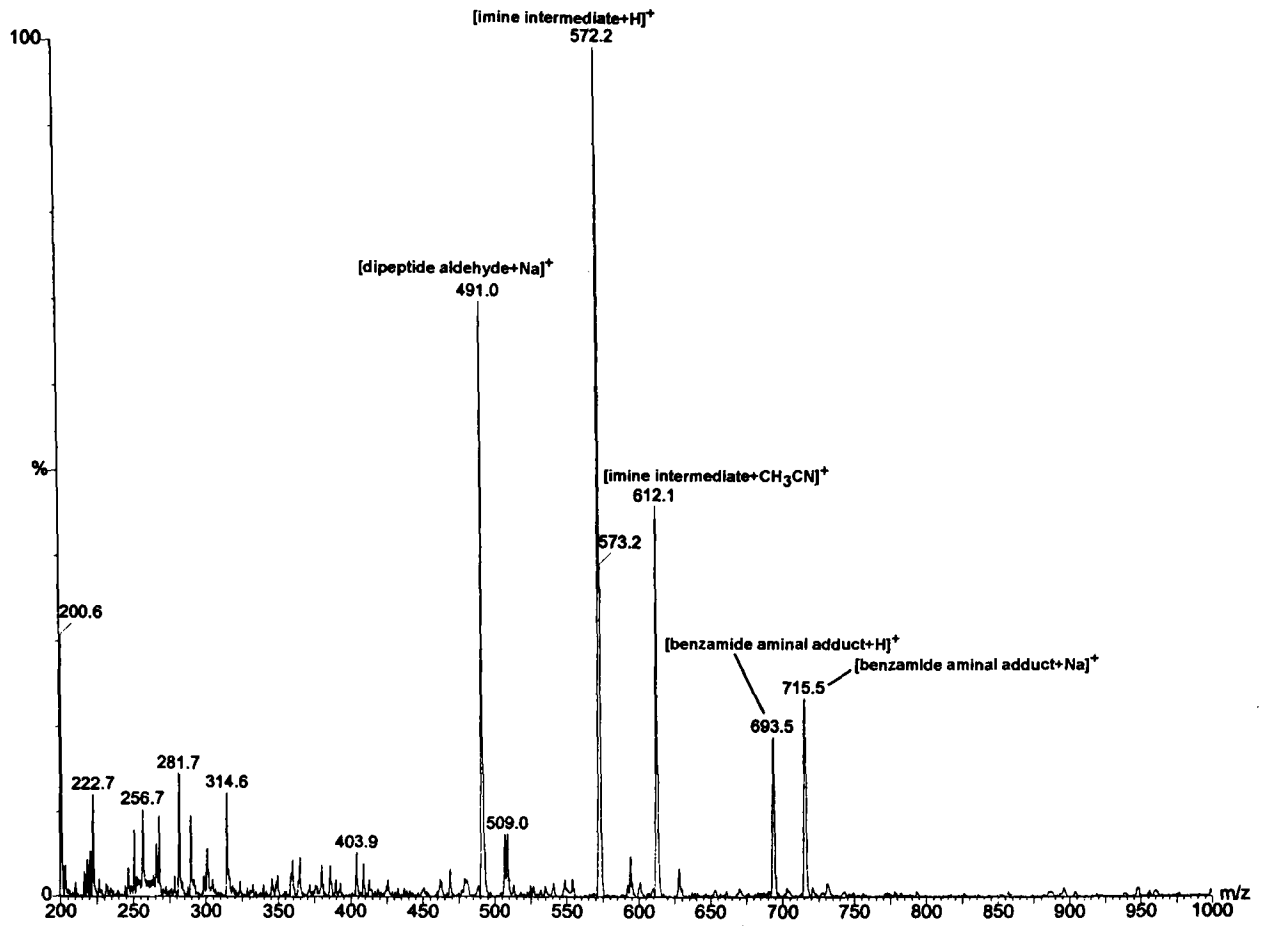
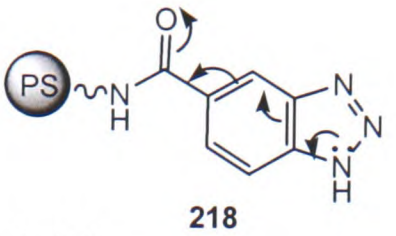


Figure 19: *ES-MS of purified dipeptide 252.*

Insolubility of the dipeptide aldehyde **252** in many common deuterated solvents hampered attempts to obtain ^{1}H NMR analysis. Eventually, a mixed solvent system ($CDCl_3/CD_3OD$) was found that sufficiently solvated the dipeptide aldehyde **252**. ^{1}H NMR of the peptide aldehyde **252** was broad and unresolved even when run on a 600 MHz instrument. However, signals were evident at 9.63 and 9.95 ppm corresponding to the diastereomeric aldehyde protons.

In view of the low/moderate yields of polystyrene bound-peptide aldehydes **249** and **250**, it was decided to optimize the peptide coupling yields. One way of achieving this, may be by changing the connectivity of the benzotriazole to the polystyrene.

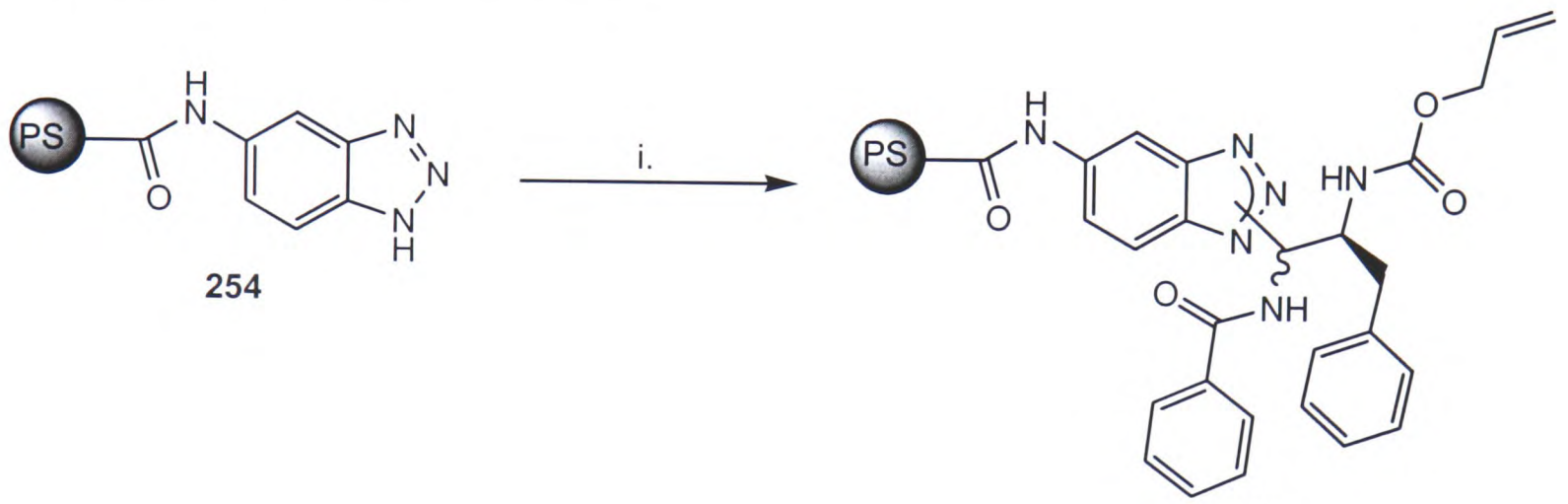
In the case of linkers **249** and **250**, benzotriazole is bound to the polystyrene backbone through an amide moiety. The conjugated amide would render the benzotriazole proton slightly more acidic and hence, the nitrogen less basic (Figure 20). Therefore, the coupling reaction would be slightly more difficult to perform on the solid- than in solution-phase.



218

Figure 20: *Weak conjugation into amide **218**.*

With this in mind, the synthesis of a benzotriazole resin **254** attached through a reversed amide functionality was carried out, using DIC coupling procedures (Scheme 66). Although other methods of attachment of benzotriazole were possible, this functionality was chosen due to the commercial availability of carboxy-polystyrene and 5-amino-benzotriazole.

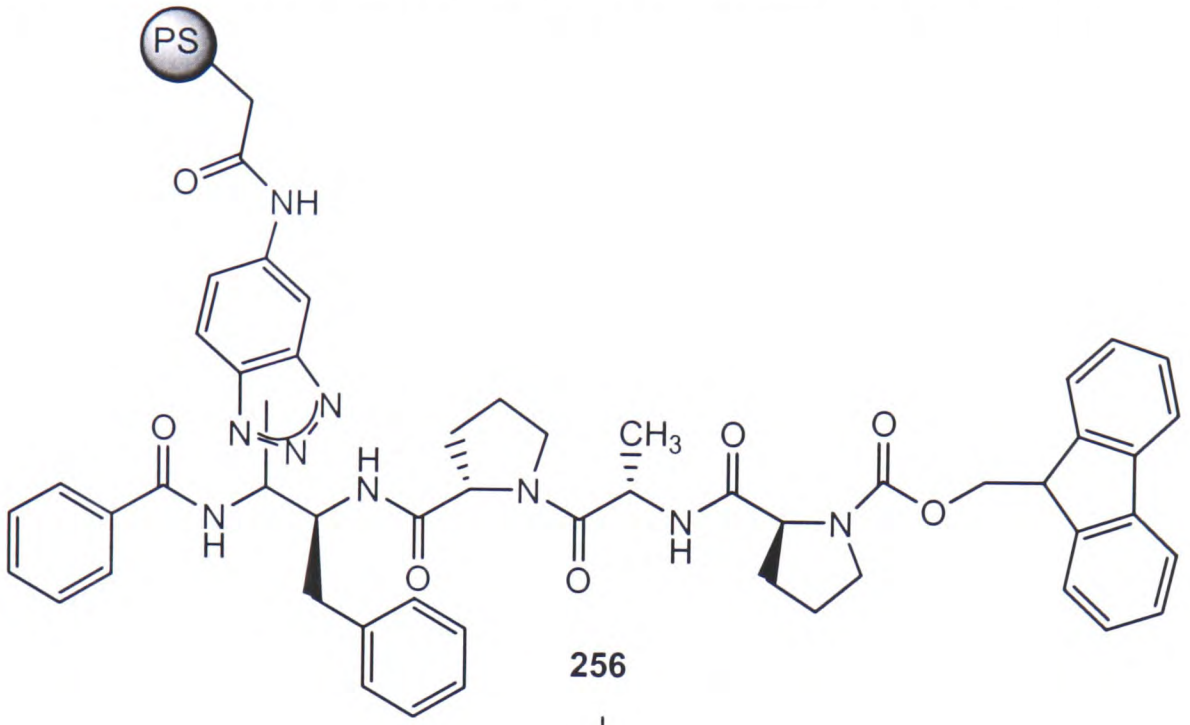


254 255

Scheme 66: *Reagents and conditions:* i. (*S*)-**171**, benzamide, *p*-toluene sulfonic acid, anhydrous toluene, 48 h, reflux, Dean-Stark apparatus.

The aminal linker **255** was generated using the same coupling procedures as before (Scheme 66). FT-IR confirmed the presence of the urethane carbonyl at 1715 cm^{-1}. MAS ^{1}H NMR was not carried out due to lack of MAS probe at the time. Peptide synthesis was carried out as before (Section 5.2) to give the polystyrene bound aldehyde **256** with a loading yield of 15%. This loading is slightly lower than the loading obtained for the conjugated amide linker, **249** (27%). One possible reason for this could be that, the now relatively basic benzotriazole nitrogen neutralizes the catalytic *p*-toluene sulfonic acid needed for the removal of water (Scheme 61). Work performed previously by Rice[82] indicated that 50% was the maximum yield obtainable for solution phase couplings using phenylacetamide (Scheme 61). Therefore, 48% loading seemed quite acceptable and no further work was carried out in this area.

Again, acidic cleavage of the peptide aldehyde **256** was performed (TFA/DCM/H_2O 9:10:1) (Scheme 67) and electrospray mass spectrometry (Figure 21) confirmed the presence of tetra-peptide aldehyde **257**. Insufficient aldehyde **257** was obtained for analysis by ^1H NMR, due to the low loading of the resin (0.091 mmol g^{-1}).



Scheme 67: *Reagents and conditions:* i. TFA/DCM/H_2O (9:10:1), 1 h, r.t.

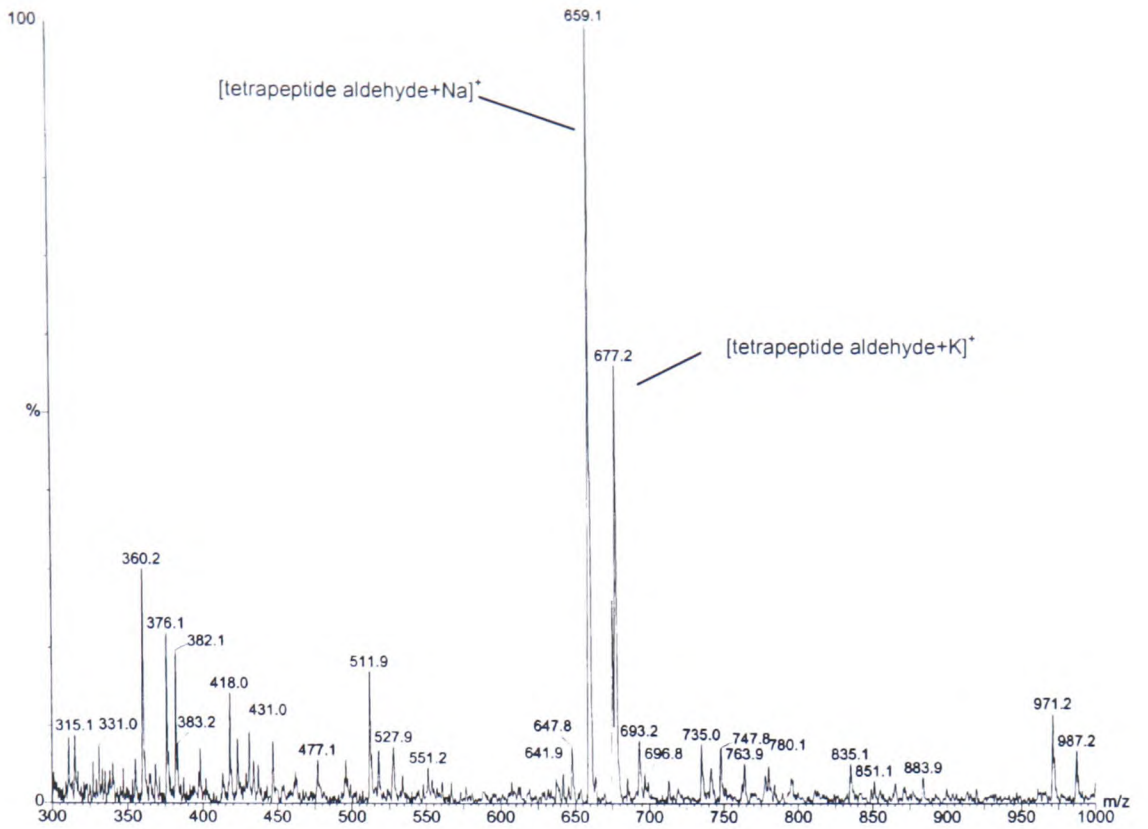


Figure 21: *ES-MS of* ***256*** *cleavage mixture.*

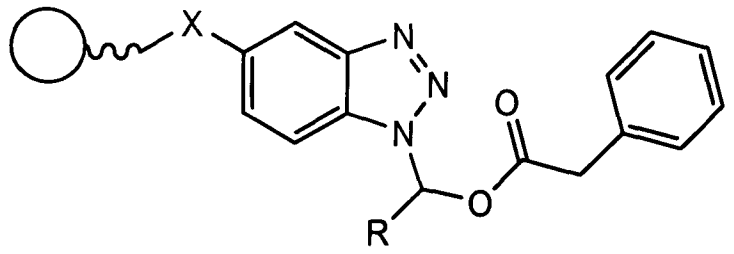
5.3 Summary and conclusions

Tetra-peptide aldehyde **257** was synthesized on polystyrene aminal linkers **250** and **256** and cleaved using acid to release **257**. Electrospray mass spectrometry of the cleavage mixtures gave the appropriate molecular ions for the tetrapeptide aldehyde **257**. Unfortunately, no ^{1}H NMR data could be obtained for the released tetrapeptide aldehyde. Tetra-peptide aldehyde **257** was also synthesized using polystyrene hemiaminal linker **328** (Chapter 7). Acidic cleavage of **328**, followed by purification using semi-preparative HPLC and electrospray mass spectrometry of the purified fraction also gave similar $[M+H]^+$, $[M+Na]^+$ and $[M+K]^+$ ions. The presence of similar molecular ion adducts from the cleavage of the three different linkers, **250**, **256** and **328** strengthens the evidence for the synthesis of tetrapeptide aldehyde **257** on polystyrene resin.

The synthesis of diastereomeric dipeptide aldehyde **252** was carried out on polystyrene aminal linker **233**. Electrospray mass spectrometry and high resolution ^{1}H NMR analysis of the acidic cleavage mixture indicated the presence of the diastereomeric dipeptide aldehyde **252** on linker **233**.

6 Results and discussion - Generation of hemiaminal linkers

As mentioned previously (Section 4.2), the synthesis of the aminal linker **233** resulted in polymerisation of the allyloxycarbonyl-phenylalaninal **171**. This was caused by the forcing conditions required for construction of the linker **233** (*i.e.* reflux in toluene for 48h with azeotropic removal of water). One way to avoid these problems would be through the use of milder reaction conditions for the generation of the linker **233**. This was envisaged through an analogous hemiaminal linker **258**, which can be generated under mild conditions[110] (Figure 22).



258

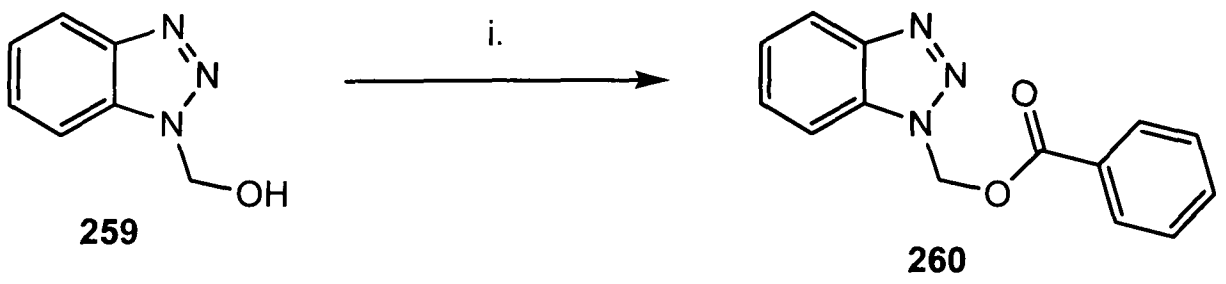
Figure 22: *Proposed structure of hemiaminal linker 258.*

6.1 Solution-phase synthesis of hemiaminal linkers

The synthesis of the hemiaminal linker **258** was first carried out in solution, with a view to eventually generating the linker on solid phase.

6.1.1 Reported syntheses of 1-(benzotriazol-1-yl)alkyl hemiaminals

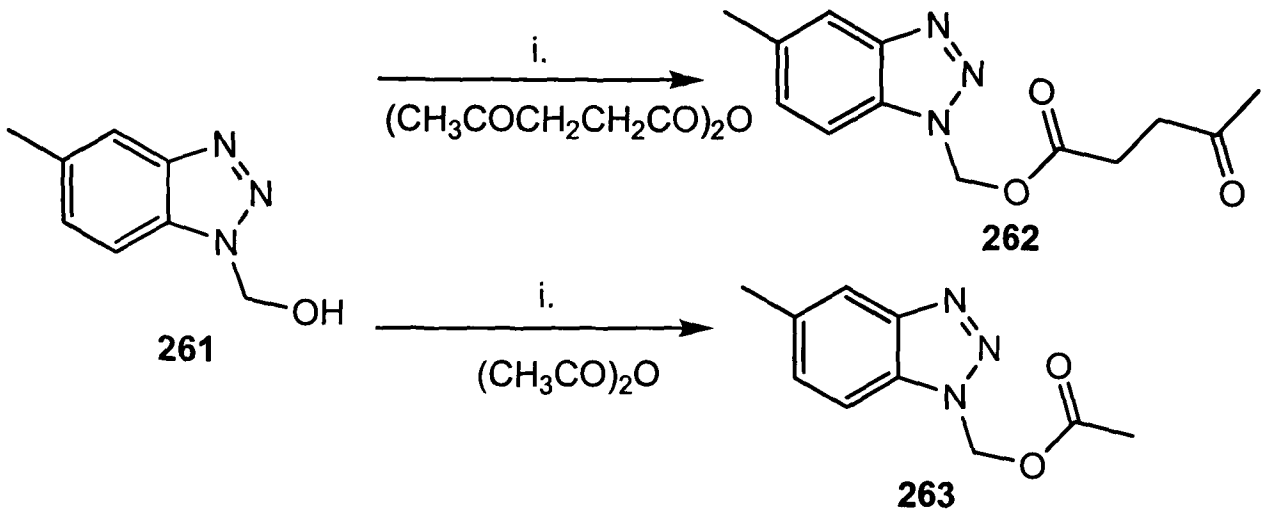
In 1957, Gaylord and Naughton[111] reported the synthesis of benzoyloxymethyl-1*H*-benzotriazole **260** by direct treatment of 1-hydroxymethyl benzotriazole **259** with benzoyl chloride or benzoic anhydride in yields of 27% and 24%, respectively (Scheme 68).



259 **260**

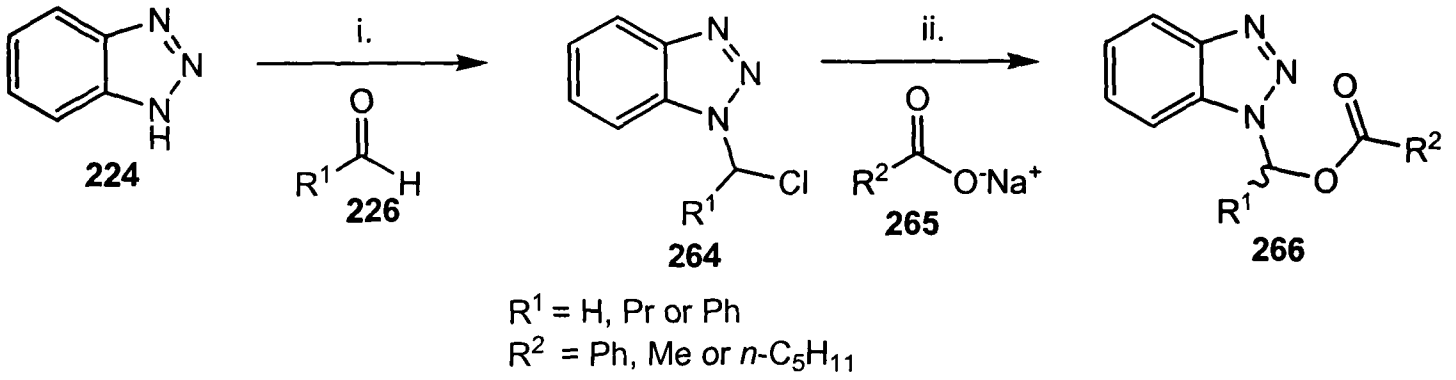
Scheme 68: *Reagents and conditions:* i. benzoyl chloride or benzoic anhydride, base.

A similar approach was taken by Ono and Itoh[112] who prepared the levulinate **262** and acetate **263** hemiaminals, by reaction of the appropriate anhydride with 5-methyl-(1-hydroxymethyl)-1*H*-benzotriazole **261** (Scheme 69).



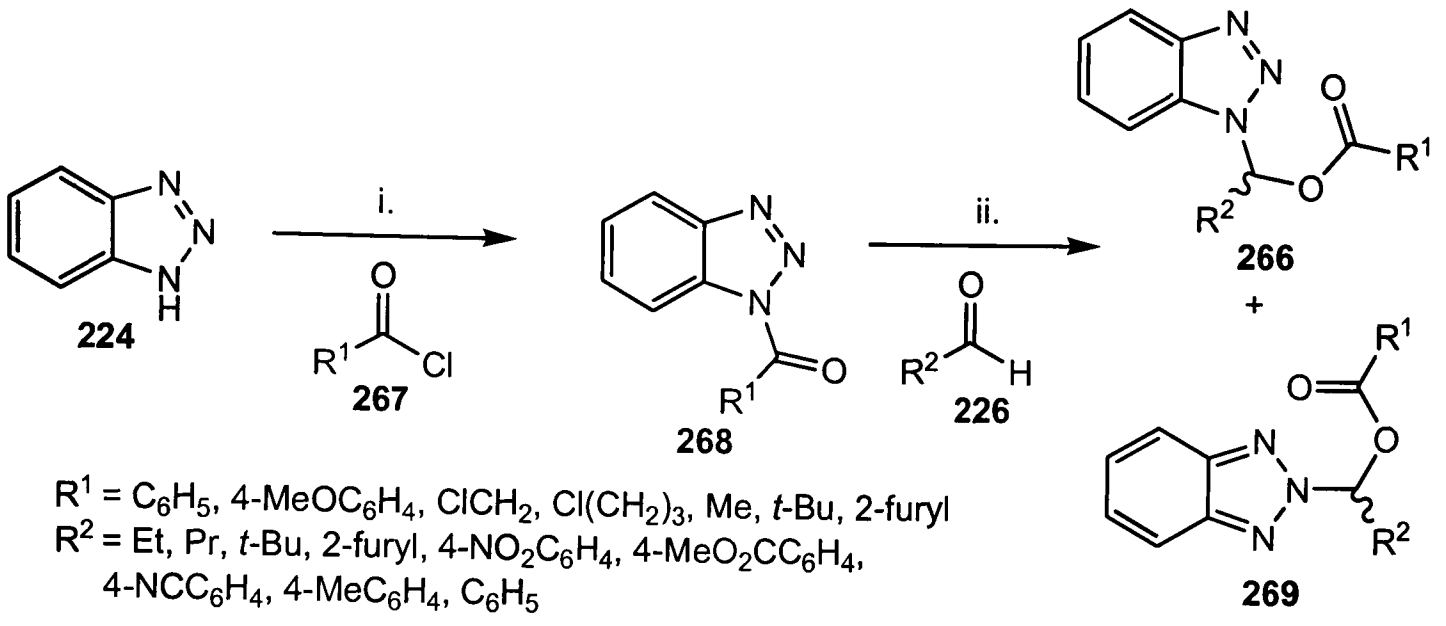
Scheme 69: *Reagents and conditions:* i. pyridine, DMAP, THF, r.t.

In 1991, Katritzky *et. al.*[113] reported the synthesis of 1-(benzotriazole-1-yl)alkyl hemiaminals **266** by the displacement of chlorine from 1-(1-chloroalkyl)benzotriazoles **264** with sodium carboxylates **265**, in yields of 71-90% (Scheme 70)



Scheme 70: *Reagents and conditions:* i. thionyl chloride, chloroform, reflux, 30 min; ii. DMSO, 50 °C, 5 h.

In a later report by Katritzky *et. al.*,[110] the synthesis of benzotriazole hemiaminals **266** and **269** by the reaction of *N*-acyl benzotriazoles **268** with either aliphatic or aromatic aldehydes **226** in acetonitrile, were described (Scheme 71).



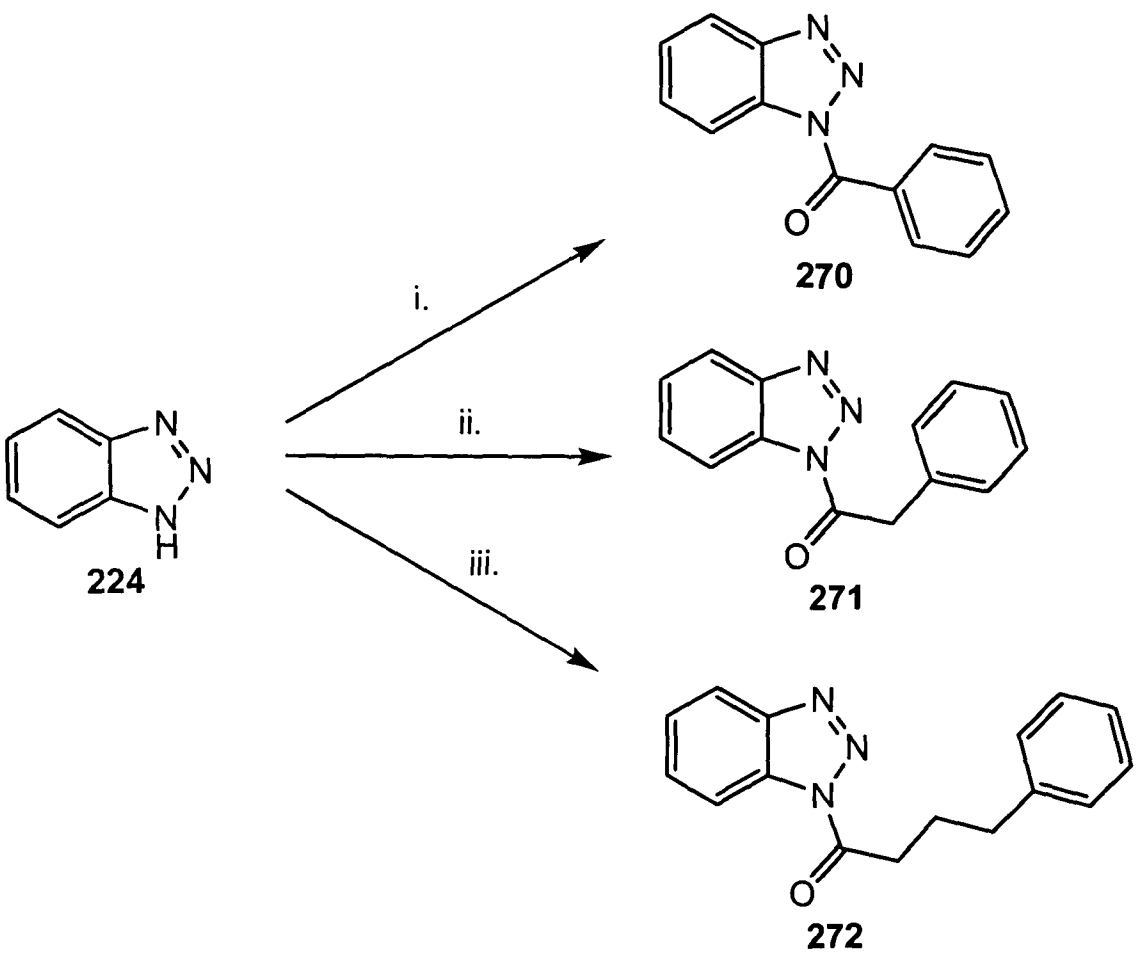
Scheme 71: *Reagents and conditions:* i. Et_3N, anhydrous DCM, 0 °C, 30 min: ii. acetonitrile, base.

Aliphatic aldehydes and electron deficient aromatic aldehydes **226** were found to give benzotriazole hemiaminals in quantitative yields at room temperature. In contrast, electron rich aromatic aldehydes **226** required elevated temperatures and prolonged reaction times. In general, most hemiaminals were obtained as the 1' isomer **266** however, some products contained small amounts (8-18%) of the 2' isomer **269**. It was also found that various bases could be used without any serious detrimental effects on the yields of hemiaminal obtained (*e.g.* potassium carbonate, triethylamine). However, formation of the 2' isomer **269** could be minimized by using triethylamine as a base. The implications of this will be discussed in more detail in Section 6.1.5.

6.1.2 Solution synthesis of model hemiaminal linkers from N-acyl benzotriazoles

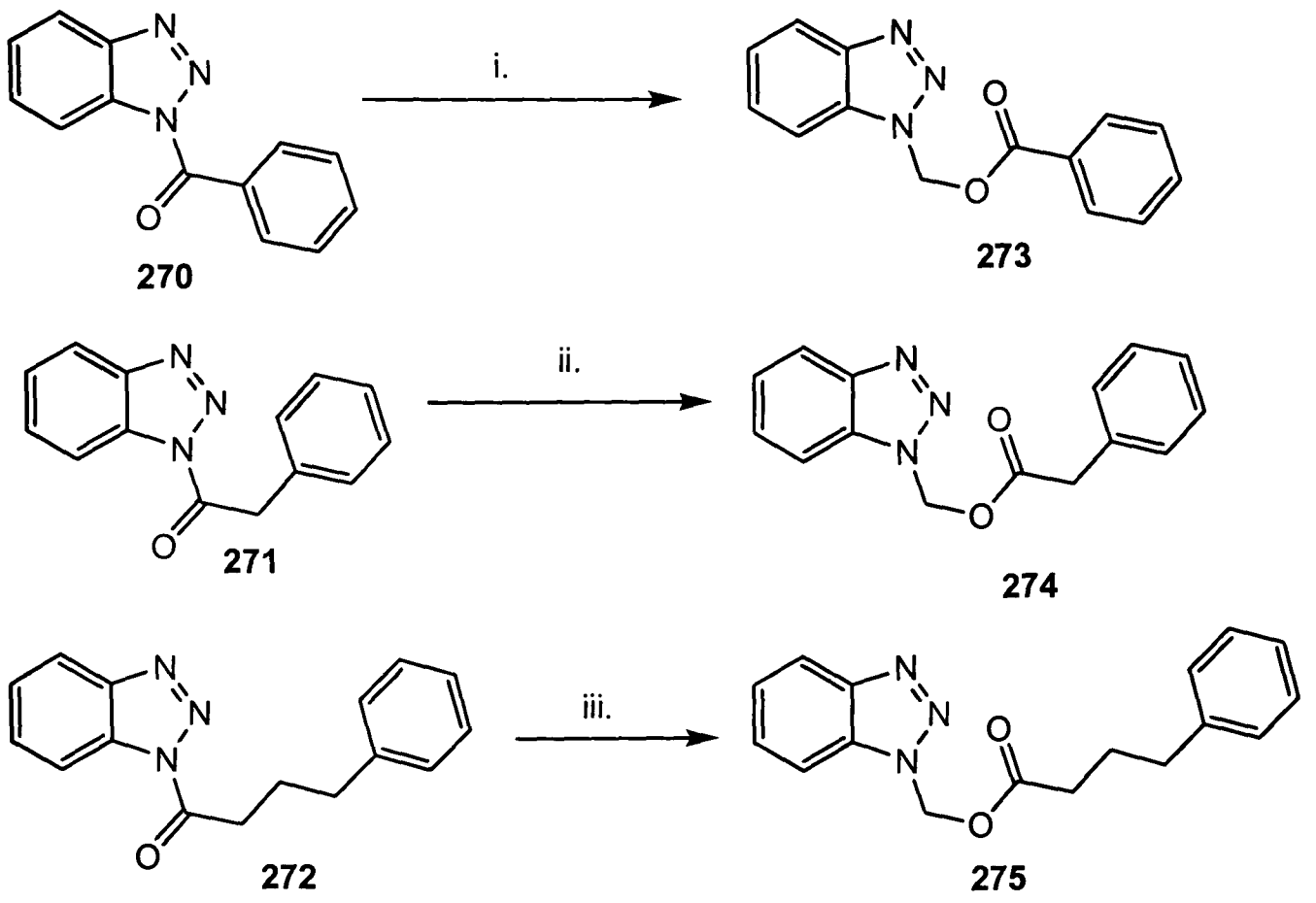
Variation of the R^2 group on aldehyde **226** allows the generation of a range of benzotriazole hemiaminals **266** and **269** from readily synthesized *N*-acyl benzotriazole **268** (Scheme 71). Using this route, a range of hemiaminals **270-272** were prepared and subjected to enzymatic hydrolysis to investigate whether they are substrates for lipases/esterases/penicillin acylase and that they fragment, according to Scheme 74, to release the corresponding acid **276**, benzotriazole **224** and aldehyde **277** into solution.

N-acyl benzotriazoles **270-272** were generated from the reaction of benzotriazole **224** with the corresponding acid chlorides in anhydrous DCM with triethylamine as base (Scheme 72). Benzoyl chloride and phenylacetyl chloride were commercially available; however 4-phenylbutyryl chloride was obtained from 4-phenylbutyric acid using oxalyl chloride with a catalytic amount of DMF (Section 8.4.1).



Scheme 72: ***Reagents and conditions***: i. benzoyl chloride, Et_3N, DCM, 56%: ii. phenylacetyl chloride, Et_3N, DCM, 73%: iii. 4-phenylbutyryl chloride, Et_3N, DCM, 71%.

N-acyl benzotriazoles **270-272** were converted to the 1' hemiaminals **273-275** by reacting with formaldehyde **277** in acetonitrile with triethylamine; the 2' hemiaminals were not detected in the reaction mixtures (Scheme 73). The yields obtained for **273-275** were lower than the quantitative yields reported by Katritzky,[110] possibly due to the presence of methanol and water in the formaldehyde solution, which could breakdown the *N*-acyl benzotriazoles **270-272**.



Scheme 73: *Reagents and conditions:* i. formaldehyde (37% in MeOH/H_2O, Et_3N, acetonitrile, r.t., 60 min, 66%; ii. formaldehyde (37% in MeOH/H_2O, Et_3N, acetonitrile, r.t., 16 h, 45%; iii. formaldehyde (37% in MeOH/H_2O, Et_3N, acetonitrile, r.t., 16 h, 63%.

6.1.3 Enzymatic hydrolysis of model hemiaminal linkers

The suitability of 1-(benzotriazole-1-yl)formyl hemiaminals **273-275** as substrates for lipase, esterase and penicillin acylase had to be ascertained. This was achieved by performing small scale hydrolysis reactions (0.3 μmol of each substrate) using 7 lipases, 1 esterase and penicillin acylase (Table 1). The substrates **273-275** were treated with each enzyme at room temperature in 10% acetonitrile/potassium phosphate buffer pH 7.4 for 16 hours and the amount of corresponding acid released **276** (Scheme 74), determined by reverse phase HPLC. Experimental conditions are given in Section 8.5.

Enzyme	**Conversion**		
	273	**274**	**275**
Control	-	-	-
Pseudomonas fluorescens lipase (PFL)	98%	98%	96%
Hog pancreas lipase (PPL)	95%	91%	90%
Chromobacterium viscosum lipase	99%	93%	95%
Candida lipolytica lipase	15%	20%	91%
Mucor javanicus lipase	35%	26%	93%
Penicillium roqueforti lipase	98%	27%	92%
Rhizopus arrhizus lipase	15%	31%	88%
Thermoanaerobium brockii esterase	99%	90%	97%
Penicillin acylase	N/A	100%	65%

Table 1: *Quantitative conversion of hemiaminal substrates **273-275** with enzymes.*

The quantitative results displayed in Table 1 indicate that all of the benzotriazole hemiaminals **273-275** were hydrolysed, at least to some extent, by all of the enzymes tested. With **273**, PFL, PPL, *Chromobacterium viscosum*, *Penicillium roqueforti* lipases and *Thermoanaerobium brockii* esterase all exhibit near quantitative conversion to the acid. In the case of **274**, PFL, PPL, *Chromobacterium viscosum* lipases and *Thermoanaerobium brockii* esterase all show near quantitative cleavage. As expected, Penicillin acylase shows quantitative conversion of **274**. In general, all the lipases cleaved **275** in almost quantitative yield, as did *Thermoanaerobium brockii* esterase. However, the conversion of **275** with Penicillin acylase was relatively poor (65%).

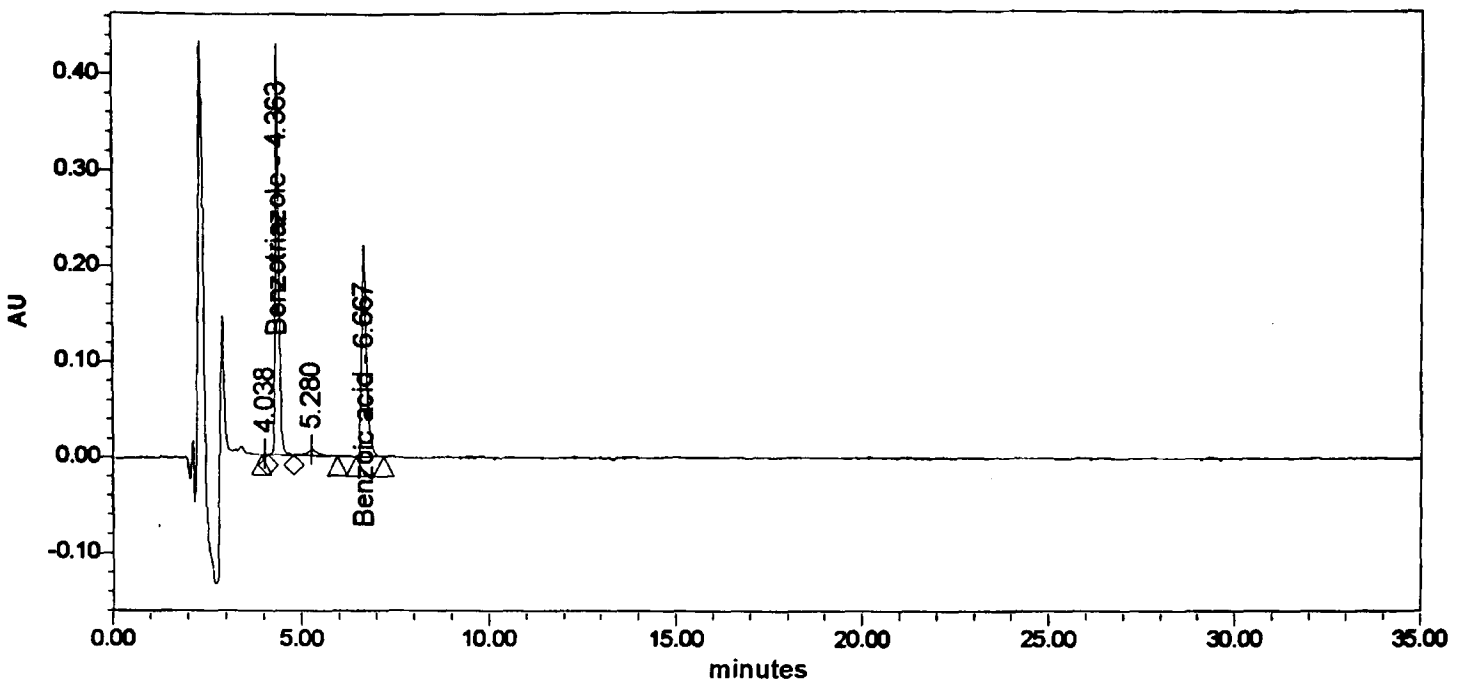
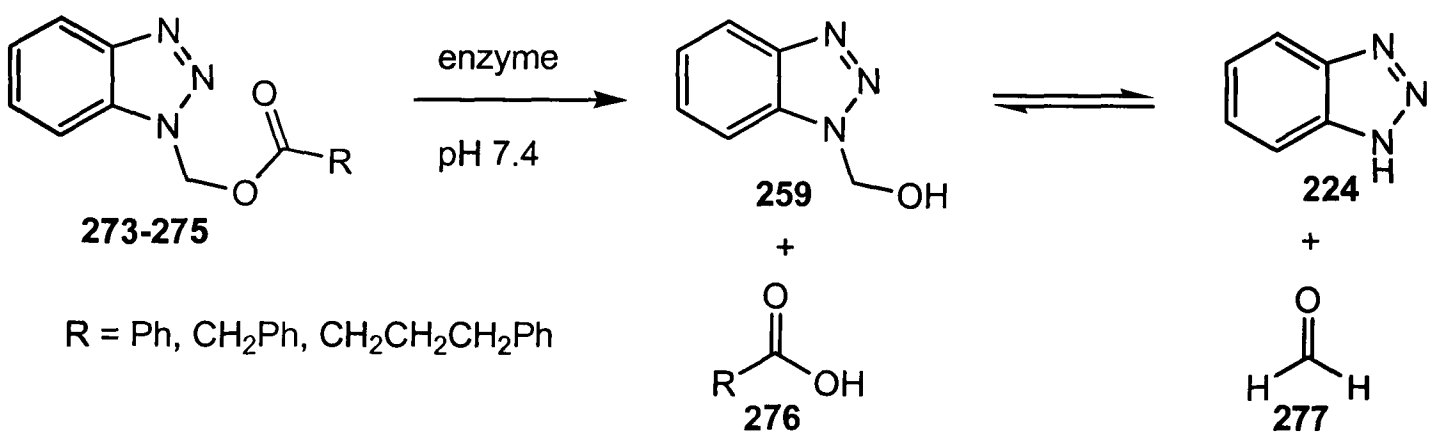


Figure 23: *Reverse phase HPLC trace of 273 cleavage with Pseudomonas fluorescens lipase.*

In addition to the corresponding acids **276**, benzotriazole **224** was detected by HPLC (Figure 23) for all these substrates indicating that the hemiacetal product **259** fragmented further to release formaldehyde **277** (Scheme 74).

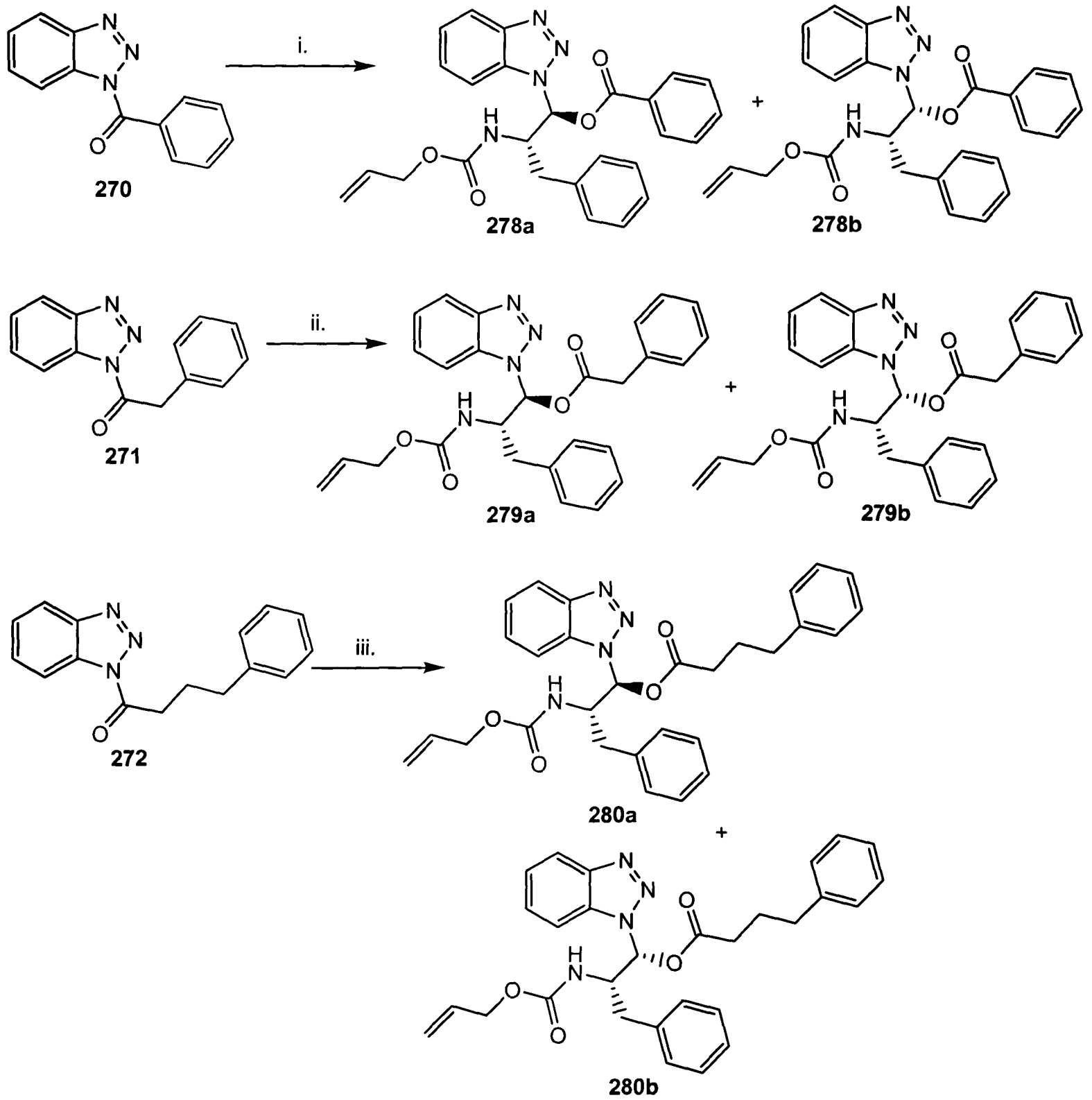


Scheme 74: *Fragmentation of 259 to formaldehyde 277 and benzotriazole 224.*

These results (Table 1) show that all of the benzotriazole hemiaminals **273-275** are substrates for the lipases, esterase and Penicillin acylase tested. Ultimately, these benzotriazole hemiaminals will be used as linkers for attachment of aldehydes, namely amino aldehydes. At this point, it was decided to generate benzotriazole hemiaminals **278-280** from (*S*)-allyloxycarbonyl-phenylalaninal **171**, to test whether these were substrates for the enzymes. It was crucial to establish that these benzotriazole hemiaminals **278-280** were substrates for these enzymes, as they contain two chiral centres and therefore, can exists as four possible diastereomers; unlike the achiral hemiaminals generated from formaldehyde **273-275**.

6.1.4 *Synthesis of diastereomeric 1-(benzotriazol-1-yl)propyl hemiaminals*

With this in mind, *N*-acyl benzotriazoles **270-272** were converted to the diastereomeric 1' hemiaminals **278-280** by reacting with (*S*)-Alloc-phenylalaninal **171** in acetonitrile or dichloromethane with triethylamine; the 2' hemiaminals were not isolated from the reaction mixtures (Scheme 73). The diastereomeric mixtures, **278a/b**, **279a/b** and **280a/b** were isolated after flash chromatography on silica gel.



Scheme 75: *Reagents and conditions:* i. (*S*)-**171**, Et_3N, acetonitrile/dichloromethane, r.t., 16 h, 55%; ii. (*S*)-**171**, Et_3N, dichloromethane, r.t., 16 h, 32%; iii. (*S*)-**171**, triethylamine, dichloromethane, r.t., 16 h, 44%.

Examination of the proton NMR for each mixture, **278-280** revealed that two diastereomers **a** and **b** were formed during the aldehyde insertion step. Both diastereomers of substrates **278-280** were observed in the RP-HPLC. In addition, small amounts (<3%) of the 2' isomers of each diastereomer were observed in the HPLC. Other bases can be used for the aldehyde insertion (*i.e.* potassium carbonate); however, Katritzky *et. al.*[110] reported that the use of triethylamine minimized the formation of the 2' isomer **269** (Scheme 71). This is favourable to limit the amount of potential isomers exposed to the enzyme and hence, allow for a more accurate substrate specificity study. The diastereomeric excess for each substrate **278a/b**, **279a/b** and **280a/b** was 45%, 21% and 22% d.e. respectively. This suggests that the aldehyde insertion step (Scheme 75) does not necessarily generate a 1:1 mixture of diastereomers. It was not deemed necessary to separate the diastereomers **a** and **b**, since no control would be possible over the diastereomeric excess of the solid phase bound analogues of these hemiaminals. Since, these diastereomers were not isolated, it is not known which diastereomer **a** or **b** is in excess.

6.1.5 Enzymatic hydrolysis of diastereomeric 1-(benzotriazole-1yl)propyl hemiaminals.

The suitability of diastereomeric 1-(benzotriazole-1-yl)propyl hemiaminals **278-280** as substrates for lipase, esterase and penicillin acylase cleavage was ascertained. This was achieved by performing small scale hydrolysis reactions (0.9 μmol of each substrate) using 7 lipases, 1 esterase and penicillin acylase (Table 2). The substrates **278-280** were treated with each enzyme at room temperature in 10% acetonitrile/potassium phosphate buffer pH 7.4 for 16 hours and the amount of corresponding acid released determined by reverse phase HPLC. Experimental conditions are given in section 8.5.

Enzyme	**Conversion (diastereomeric excess of unhydrolysed hemiaminals)**		
	278a/b	**279a/b**	**280a/b**
Control	- (45%)	- (21%)	- (22%)
Pseudomonas fluorescens lipase (PFL)	-	1.5%	-
Hog pancreas lipase (PPL)	-	20% (62%)	72% (64%)
Chromobacterium viscosum lipase	-	1.4%	1.6%
Candida lipolytica lipase	1.4% (42%)	1.4%	-
Mucor javanicus lipase	-	1.4%	-
Penicillium roqueforti lipase	-	1.5%	2.6%
Rhizopus arrhizus lipase	-	-	-
Thermoanaerobium brockii esterase	-	3.6%	-
Penicillin acylase	-	27% (20%)	-

Table 2: *Conversion of diastereomeric benzotriazole hemiaminals **278-280** with enzyme.*

In all reactions where hydrolysis was observed, both (*S*)-Alloc-phenylalaninal **171** and benzotriazole **224** were observed in the reverse phase-HPLC traces (R_t = 4.4 min and 9.6 min respectively). From Table 2, it can be seen that neither of the diastereomers of benzotriazole hemiaminal **278a/b** were hydrolysed by any of the enzymes tested. In the case of **279a/b**, Hog pancreas lipase and Penicillin acylase cleaved the substrate in only moderate yields (20% and 27% respectively) (Figure 24).

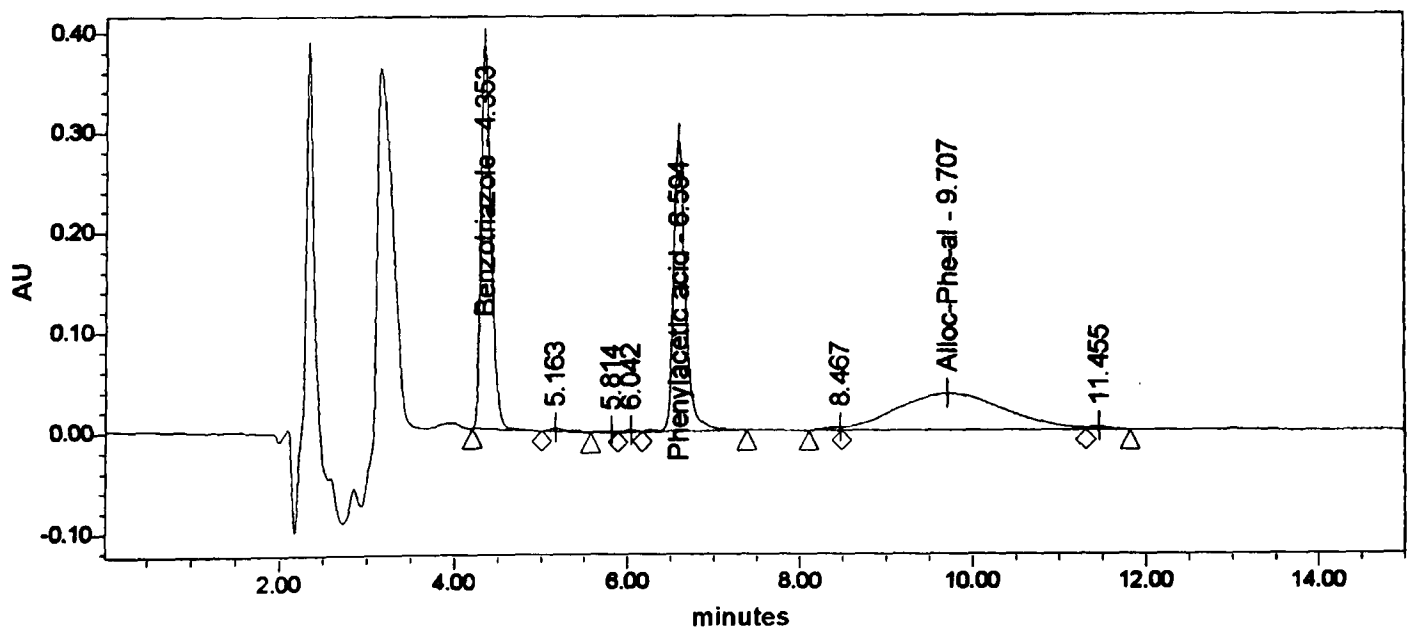


Figure 24: *Reverse phase HPLC trace of **279a/b** cleaved with Penicillin acylase.*

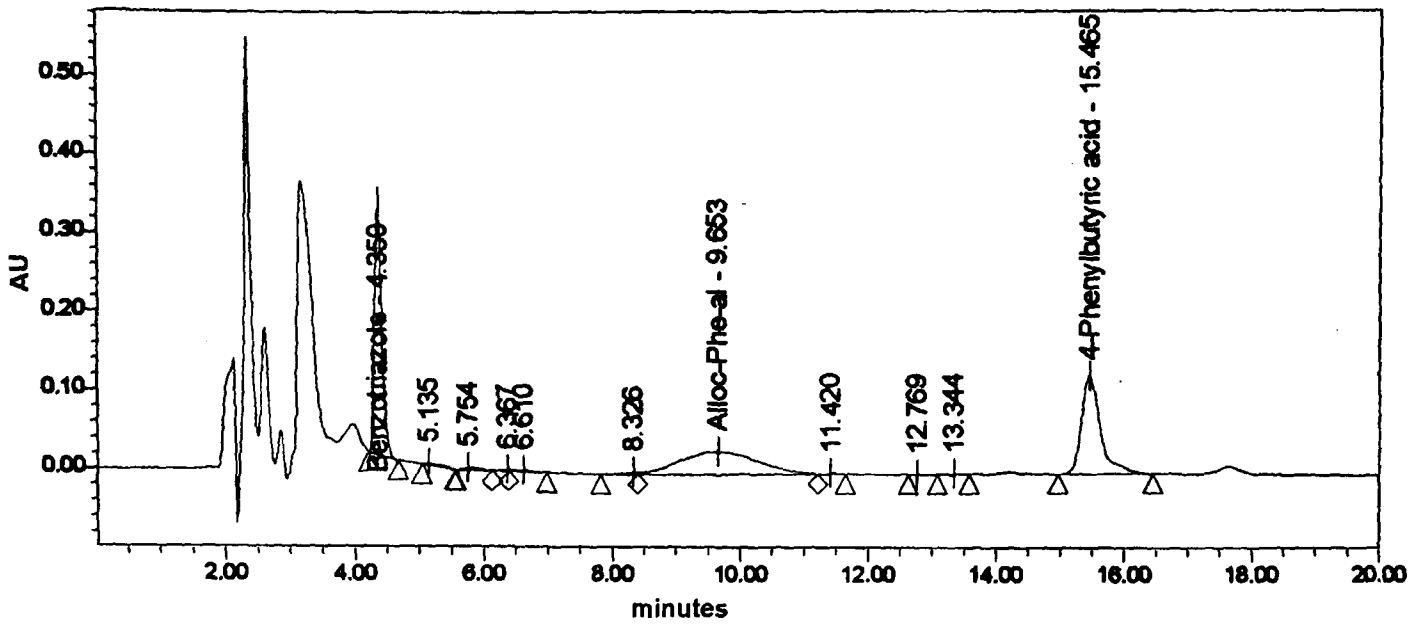


Figure 25: *Reverse phase HPLC trace of **280a/b** cleaved with Hog pancreas lipase.*

The control experiment exhibited a diastereomeric excess of 21% for unhydrolysed **279a/b**; whereas, in the presence of Hog Pancreas lipase a diastereomeric excess of 62% was obtained for unhydrolysed **279a/b**. This showed that Hog pancreas preferencially hydrolyses one diastereomer over the other. On the other hand, Penicillin acylase did not seem to exhibit any diastereoselectivity (*i.e.* 20% d.e. *c.f.* 21% d.e. in control). For **280a/b**, only Hog Pancreas lipase cleaved the hemiaminal in good yield (*i.e.* 72%) (Figure 25) and was shown to be selective for the minor diastereomer (*i.e.* diastereomeric excess of the unhydrolysed material increased to 64%) (Figure 26).

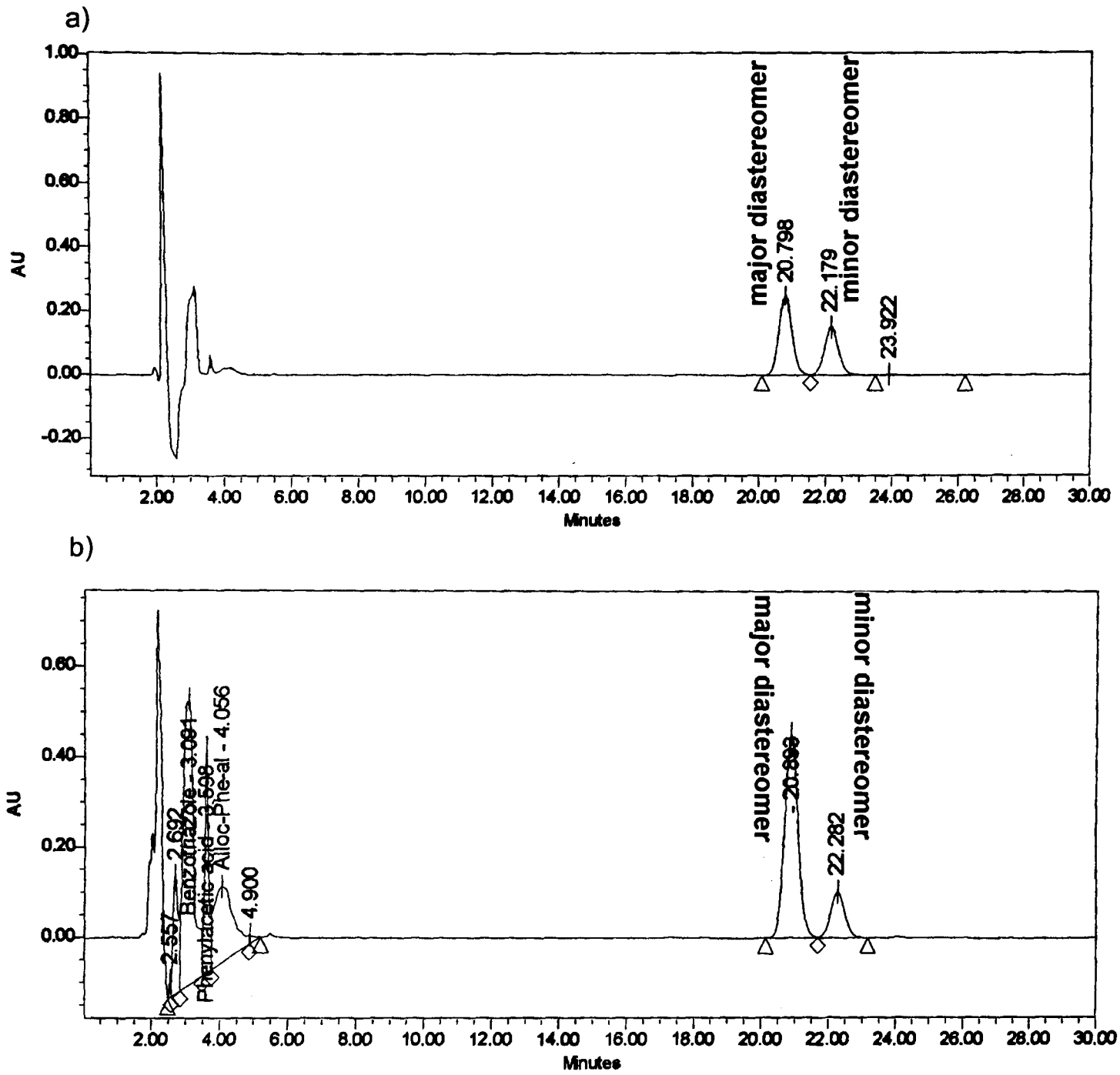


Figure 26: *Reverse phase HPLC trace of a) Control containing **279a/b** and b) **279a/b** cleaved with Hog pancreas lipase.*

6.1.6 Summary and conclusions

Model 1-(benzotriazol-1-yl)alkyl hemiaminals **273-275** were synthesized using methodology developed by Katritzky *et. al.*[110] (45-66% yield). Enzymatic screening revealed that all of the benzotriazole hemiaminals **273-275** were substrates for all of the lipases and the esterase. In general, hemiaminal **275** was the best substrate for the lipases and hemiaminal **274** was the best substrate for penicillin acylase.

Diastereomeric 1-(benzotriazole-1-yl)propyl hemiaminals **278a/b-280a/b** were prepared in order to study the effect of the diastereomeric centres on the substrate specificity of the enzymes. In the case of **278a/b**, none of the lipases or the esterase

hydrolysed the diastereomeric hemiaminal **278a/b**. This is in sharp contrast to the achiral benzotriazole hemiaminal equivalent **273** where nearly all the lipases/esterase hydrolysed the substrate **273** in excellent yield (>90% conversion). Only penicillin acylase and Hog pancreas lipase hydrolysed the diastereomeric hemiaminal **279a/b** in 27 and 20% respectively. In addition, Hog pancreas preferentially hydrolyses one diastereomer over the other (62% d.e.). For **280a/b**, only Hog pancreas lipase cleaved the hemiaminal in good yield (72%) and diastereomeric excess (64% d.e.).

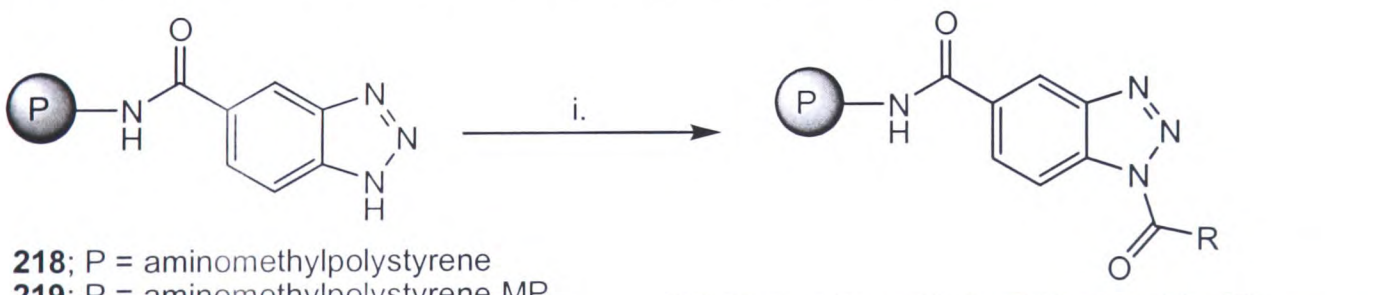
To conclude, it can be seen that the presence of the allyloxycarbonyl-amino-3-phenyl-propyl group in substrates **278-280** dramatically decreases the substrate specificity of all the enzymes tested, compared to the methyl hemiaminals **273-275**.

6.2 Solid phase synthesis of hemiaminal linkers

With the results of the enzymatic hydrolysis of the solution phase analogues **273-275** and **278-280** in mind, the synthesis and enzymatic hydrolysis of the solid support bound equivalents were undertaken.

6.2.1 Solid phase synthesis N-acyl benzotriazole resins

Polymer bound *N*-acyl benzotriazoles **281-286,** were prepared by acetylation of benzotriazole adducts **218-220** with the corresponding acid chlorides in anhydrous dichloromethane with triethylamine as base (Scheme 76).



218; P = aminomethylpolystyrene
219; P = aminomethylpolystyrene MP
220; P = $PEGA_{1900}$

281; P = aminomethyl polystyrene, R = Ph
282; P = aminomethyl polystyrene MP, R = Ph
283; P = aminomethyl polystyrene MP, R = CH_2Ph
284; P = $PEGA_{1900}$, R = Ph
285; P = $PEGA_{1900}$, R = CH_2Ph
286; P = $PEGA_{1900}$, R = $CH_2CH_2CH_2Ph$

Scheme 76: *Reagents and conditions:* i. benzoyl chloride, phenylacetyl chloride, or 4-phenylbutyric chloride, Et_3N, anhydrous dichloromethane, 20 h, r.t.

Formation of the *N*-acyl bond resulted in a strong carbonyl stretch at ~1700 cm^{-1} in the FT-IR for all polymer bound substrates **281-286**. For substrates **281** and **284-286**, additional aromatic signals can be seen in the MAS ^{1}H NMR spectra which can be attributed to the extra phenyl rings present in the polymer bound *N*-acyl benzotriazole (Figure 27).

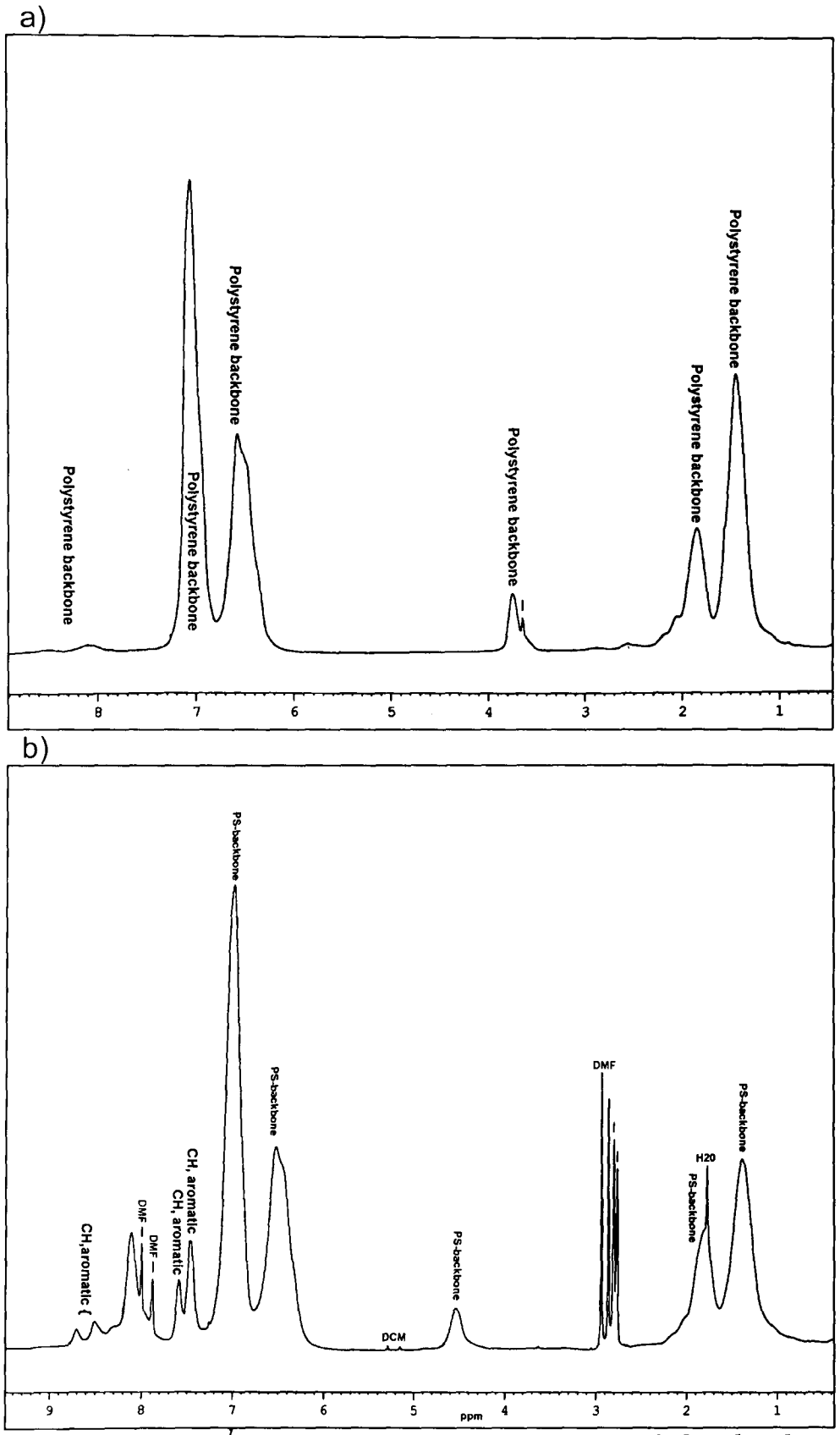
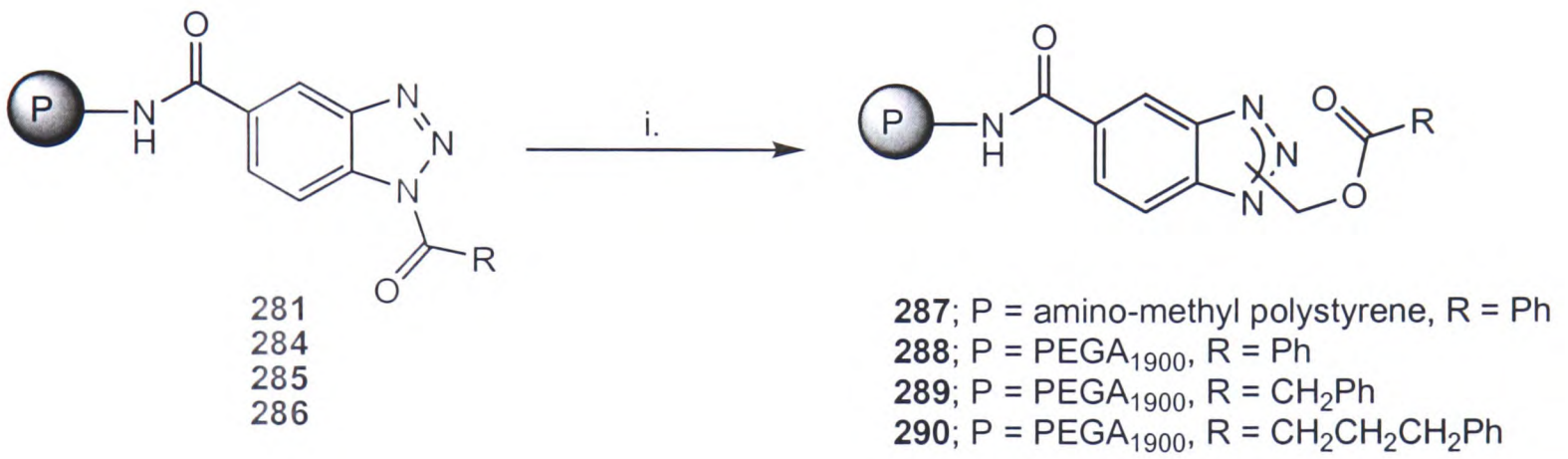


Figure 27: *High resolution MAS ^{1}H NMR spectrum of a) aminomethylated polystyrene **214** and b) **281**.*

The polymer bound *N*-acyl benzotriazoles **281-286** were found to be relatively unstable at room temperature decomposing over a few weeks but could be stored for several months below 0 °C.

6.2.2 *Solid-phase synthesis of model hemiaminal linker*

The preparation of polymer-bound benzotriazole hemiaminals **287-290** was carried out by reacting *N*-acyl benzotriazole resin **281, 284-286** with formaldehyde **277** in acetonitrile with triethylamine as base, for 20h at room temperature (Scheme 77).



Scheme 77: *Reagents and conditions:* i. polymer-bound *N*-acyl benzotriazole, formaldehyde, Et_3N, acetonitrile, 20 h, r.t.

The loading for substrate **287** was much improved over the aminal linker analogue **232** (*i.e.* 77%, *c.f.* 58% loading). This was probably due to the milder conditions required for the synthesis of **287** compared to conditions required for the condensation of benzotriazole, formaldehyde **277** and benzamide for the synthesis of **232**. It should be noted that $PEGA_{1900}$ linker **288** was obtained with a loading of 34%, which is substantially less than the equivalent polystyrene linker **287** (77%). This was probably caused by a greater affinity of the organic reagents/solvents for the hydrophobic polystyrene resin **287**.

For each substrate **287-290**, the complete disappearance of the *N*-acyl carbonyl stretch at 1700 cm^{-1} and the emergence of an ester carbonyl stretch at ~1730 cm^{-1} in the FT-IR indicated complete insertion of the formaldehyde **277** into the *N*-acyl bond. Additionally, an extra peak at ~6-7 ppm was evident in all MAS ^1H NMR

spectra which was assigned to the hemiaminal methylene of substrates **287-290** (Figure 28).

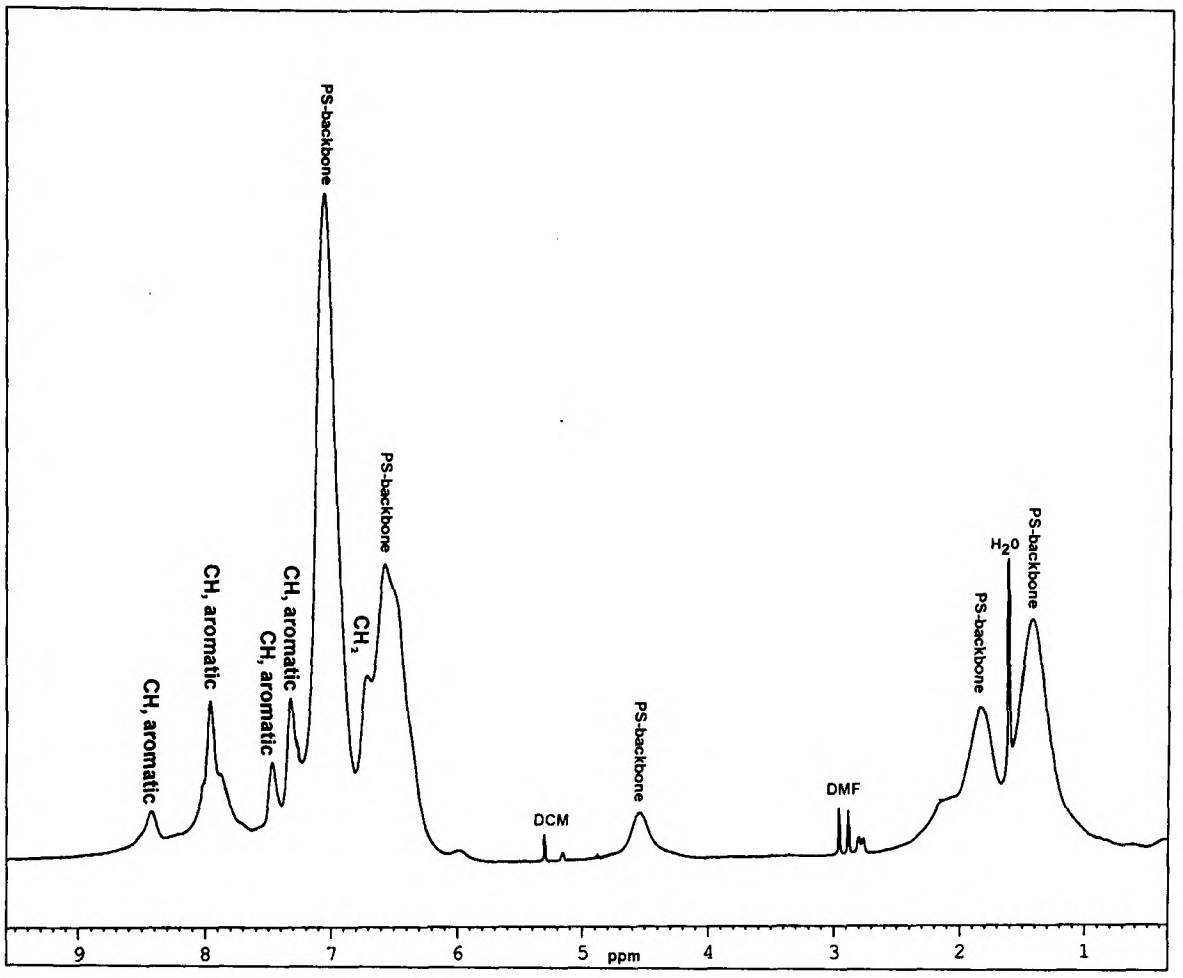


Figure 28: *High resolution MAS ^{1}H NMR spectrum of **287**.*

It is assumed that a mixture of 1', 2' and 3' hemiaminals were formed, although it was thought that the 1' hemiaminal was the predominant form (Figure 29).

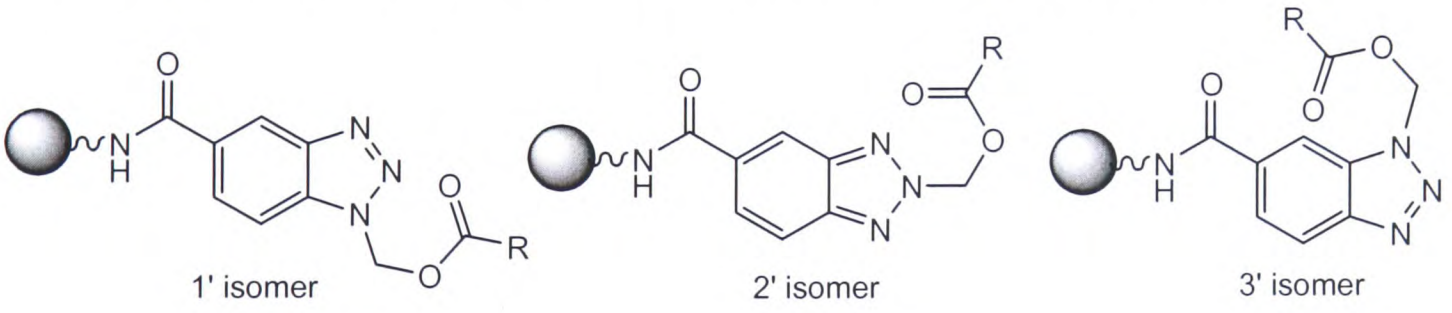
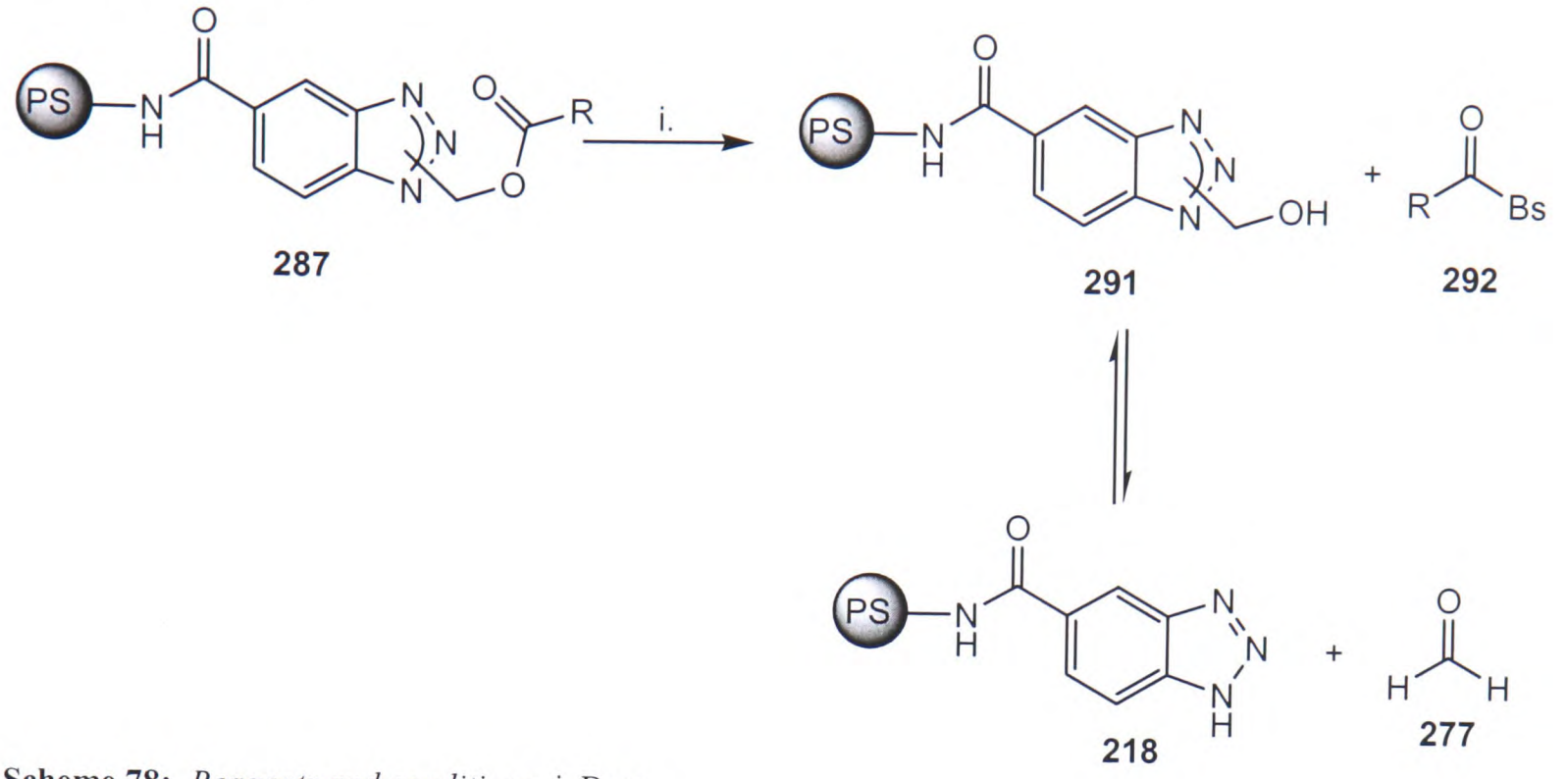


Figure 29: *1', 2' and 3' isomers of benzotriazole hemiaminals **287-290**.*

6.2.3 Evaluation of chemical cleavage methods for model hemiaminal linker

The ability to cleave polymer bound benzotriazole hemiaminals **287-290** is central to their application as effective aldehyde linkers. The cleavage was envisaged through base induced hydrolysis of the ester **287**, followed by fragmentation of the mixed hemiacetal moiety **291** (Scheme 78). Polystyrene-bound

benzotriazole hemiaminal **287,** was chosen as a simple model system to test various cleavage methods.



Scheme 78: *Reagents and conditions:* i. Base.

A selection of bases were screened for their effectiveness in the cleavage of polystyrene bound benzotriazole hemiaminal **287**. The substrate was treated with each base according to the experimental procedures detailed in Section 8.6.27 and the amount of acid derivative released determined by HPLC (Table 3).

Base	**Conversion**
7N NH_3/MeOH	++++
5% hydrazine hydrate/DMF	++++
Diisopropylethylamine/MeOH/DMF (1:5:5)	++
1M sodium methoxide/Dioxane	+
1M sodium hydroxide/Dioxane	++++

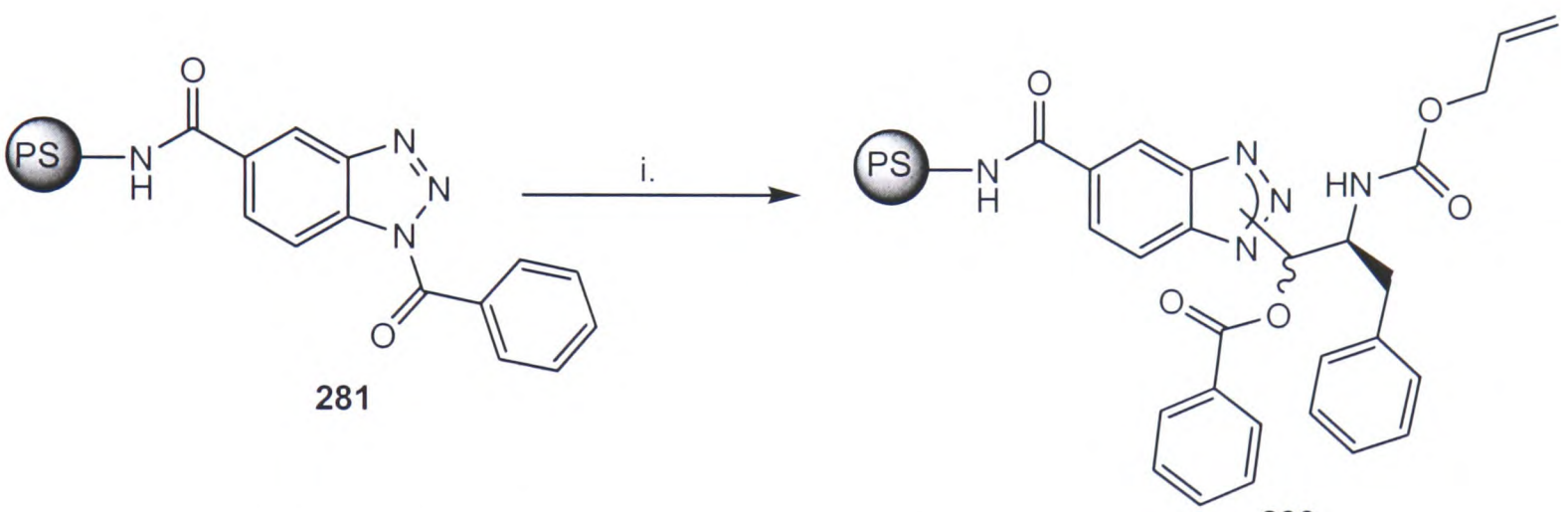
Table 3: *+(0-25%), ++(26-50%), +++(51-75%), ++++(76-100%).*

The qualitative results displayed in Table 3 indicate that methanolic ammonia, hydrazine hydrate and sodium hydroxide cleave the ester **287**, in almost quantitative yield (no further cleavage was observed after re-treating the resins with each base followed by HPLC).

Ultimately, the benzotriazole hemiaminal linker **287** was to be used for the attachment, manipulation and subsequent release of amino aldehydes into solution. Therefore, it was essential to ensure that the cleavage methods (Table 3) were suitable for the release of aldehydes into solution.

6.2.4 Solid phase synthesis of diastereomeric hemiaminal linkers

With the results of the base cleavage of **287** in mind, the synthesis of Alloc-protected phenylalaninal coupled benzotriazole resin **293** was undertaken. This was achieved by reacting *N*-acyl benzotriazole resin **281** with (*S*)-allyloxycarbonyl-phenylalaninal **171** in acetonitrile with triethylamine as base (Scheme 79).



Scheme 79: *Reagents and conditions:* i. (*S*)-**171**, Et_3N, acetonitrile, 20 h, r.t.

Analysis of **293** by FT-IR revealed the formation of an ester carbonyl stretch at 1730 cm^{-1}. In addition, the MAS ^1H NMR spectrum of **293** showed characteristic signals at 4.54, 5.06 and 5.75 ppm consistent with the protons of the allyloxycarbonyl-protecting group (Figure 30).

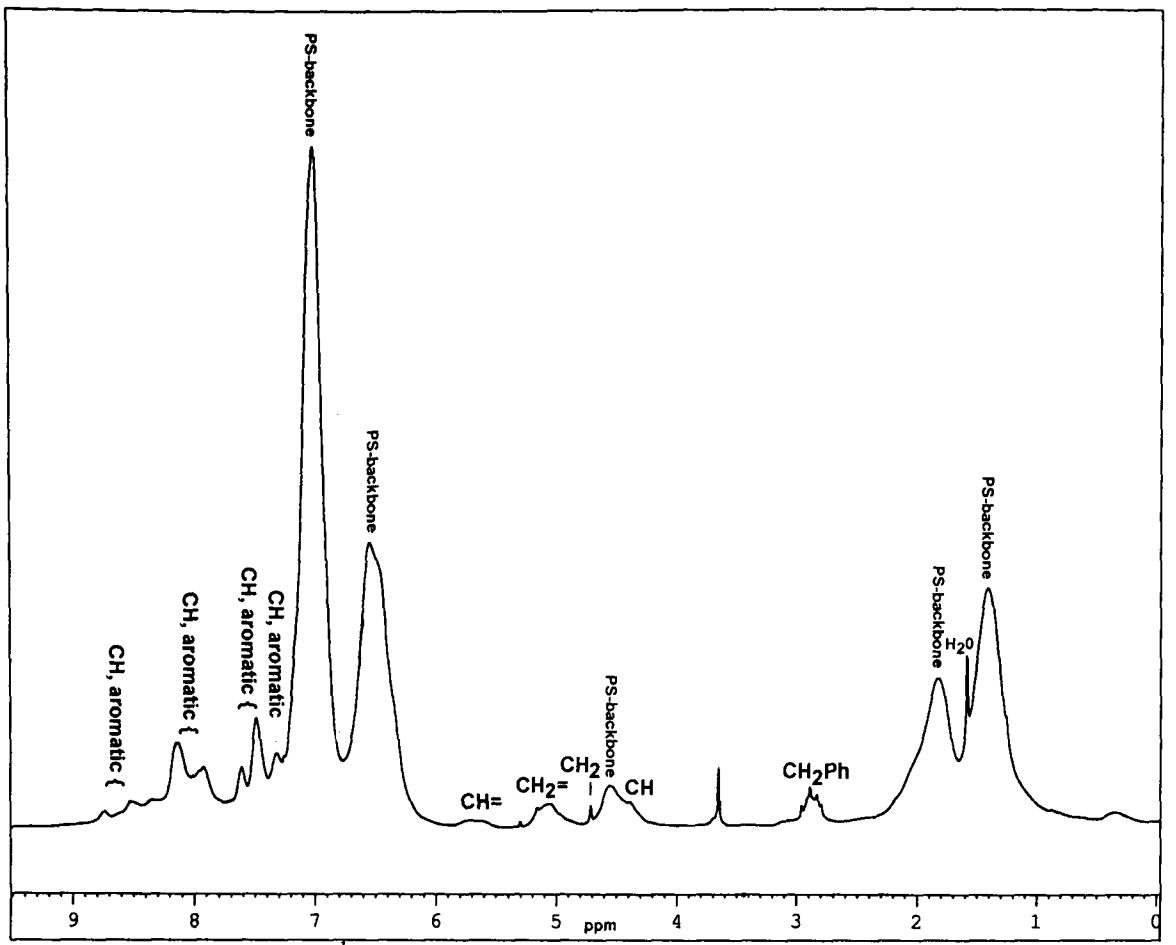
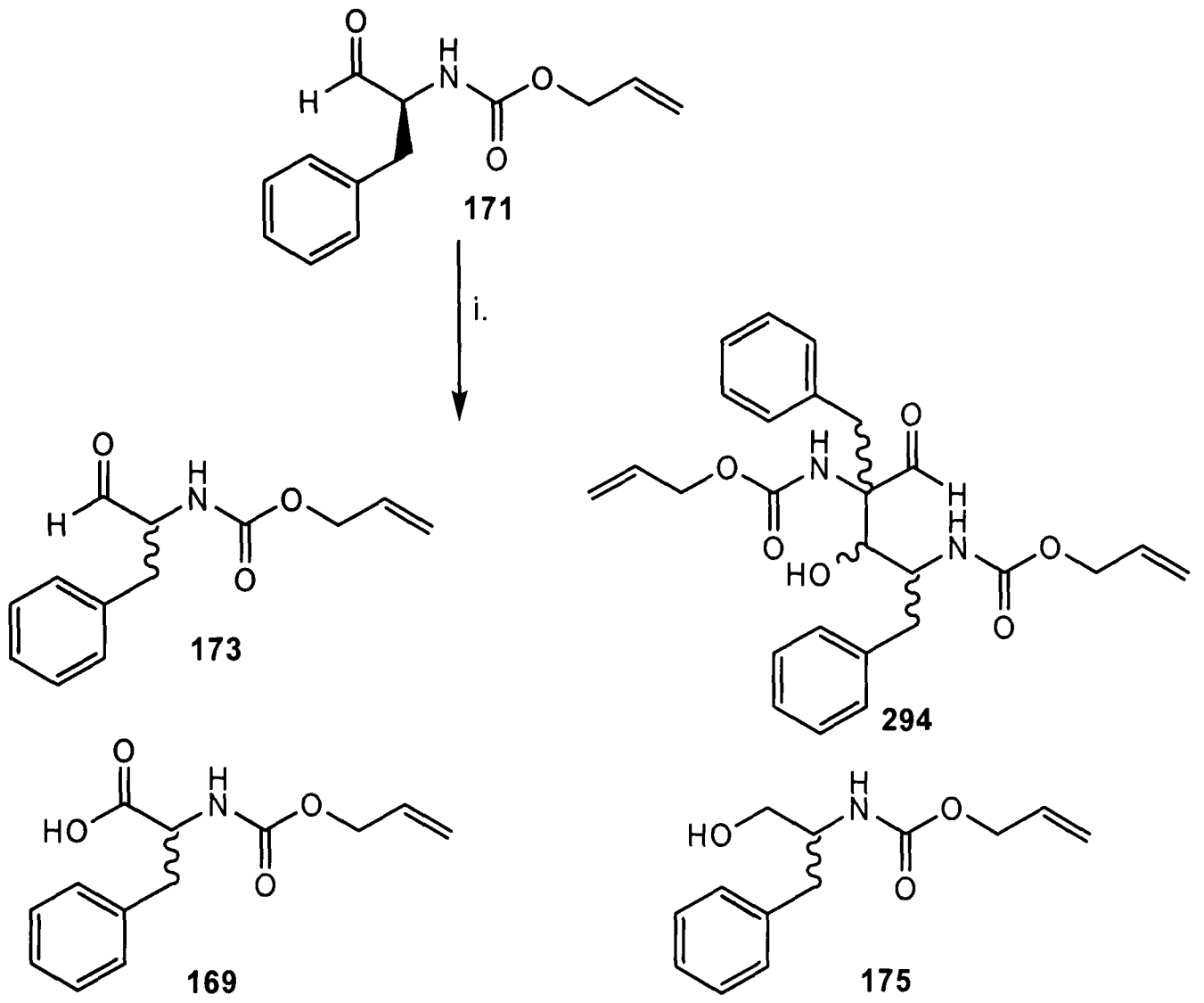


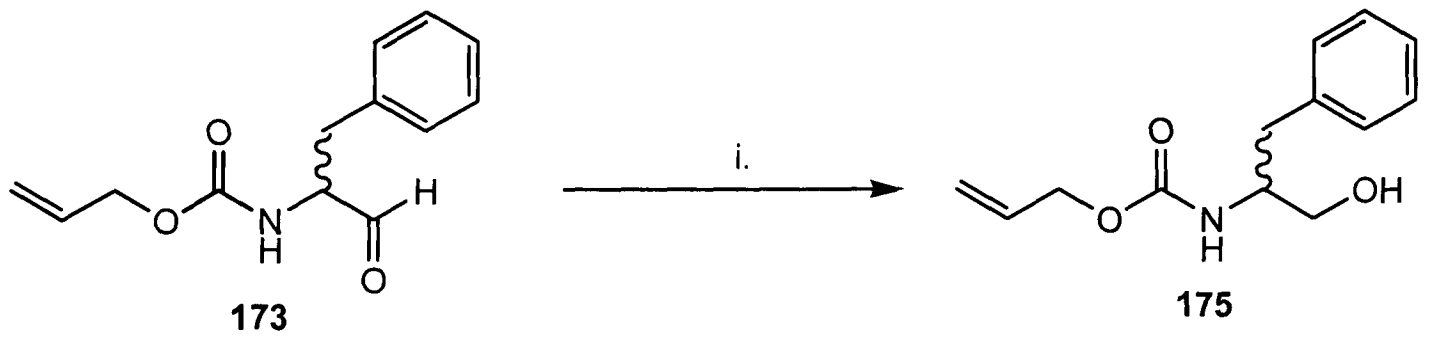
Figure 30: *High resolution MAS ^{1}H NMR of **293**.*

With **293** in hand, base cleavages were repeated as before and the samples analysed by electrospray mass spectrometry according to Section 8.2.8. It was found that although the bases listed in Table 3 cleaved linker **293**, to release the corresponding acid derivative, only methanolic ammonia and sodium hydroxide released the desired allyloxycarbonyl-phenylalaninal **171/173** into solution. It should be noted that allyloxycarbonyl-phenylalaninal **171/173** was unstable in sodium hydroxide since treatment with a solution of 1 M sodium hydroxide in dioxane for 2 hour resulted in a mixture of allyloxycarbonyl-phenylalaninal **173**, self-condensation aldol product **294**, acid **169** and alcohol **175** as identified by electrospray mass spectrometry (Scheme 80).



Scheme 80: *Reagents and conditions:* i. 1 M NaOH/dioxane (3:2), 2 h.

An aliquot of the cleaved allyloxycarbonyl-phenylalaninal **173** was reduced using sodium borohydride (Scheme 81) and analysed by normal phase chiral HPLC according to Section 8.2.9.



Scheme 81: *Reagents and conditions:* i. Sodium borohydride, EtOAc, r.t., 30 min.

Not surprisingly, it was found that the cleaved allyloxycarbonyl-phenylalaninal **173** was completely epimerized (0% e.e.) during sodium hydroxide and methanolic ammonia cleavage. One way of avoiding the problem of epimerization of the cleaved aldehyde could be through the use of hydrolytic enzymes.

6.2.5 *Enzymatic hydrolysis of model enzyme labile PEGA$_{1900}$-bound hemiaminal linkers*

As discussed previously in Section 6.2.2 (Scheme 77), PEGA$_{1900}$-bound benzotriazole hemiaminals **288-290** were prepared and enzyme cleavage studies were carried out by Dr. Rein Ulijn at Edinburgh University. Small scale hydrolysis reactions were carried out (20 mg of resin) using 5 lipases. Each substrate was treated with each enzyme at room temperature in 10% acetonitrile/potassium phosphate buffer pH 7.4 for 16 hours and the amount of corresponding acid released was quantified by reverse phase HPLC (Section 8.2.9). All experimental details are described in Section 8.6.37. Although, most of the lipases selected for this experiment were different to those chosen to cleave the solution phase analogues **273-275** (Scheme 73); they were selected to give a range of enzymes with different molecular weights. The results for Hog pancreas lipase cleavage were compared (Table 4). In all of the solution phase analogues, Hog pancreas lipase cleaved substrates **273-275**, in excellent yield (>90%). With the PEGA$_{1900}$-bound substrates, only **288** was hydrolysed by Hog pancreas lipase in moderate yield (42%); whereas, **289** and **290** were not cleaved to any significant extent by Hog pancreas lipase (5 and 0% respectively). It would seem that enzymatic cleavage in the solution phase does not guarantee enzymatic cleavage from the solid-phase analogues and therefore, direct comparisons between solution- and solid-phase analogues cannot be made in the case of enzymatic hydrolyses yields.

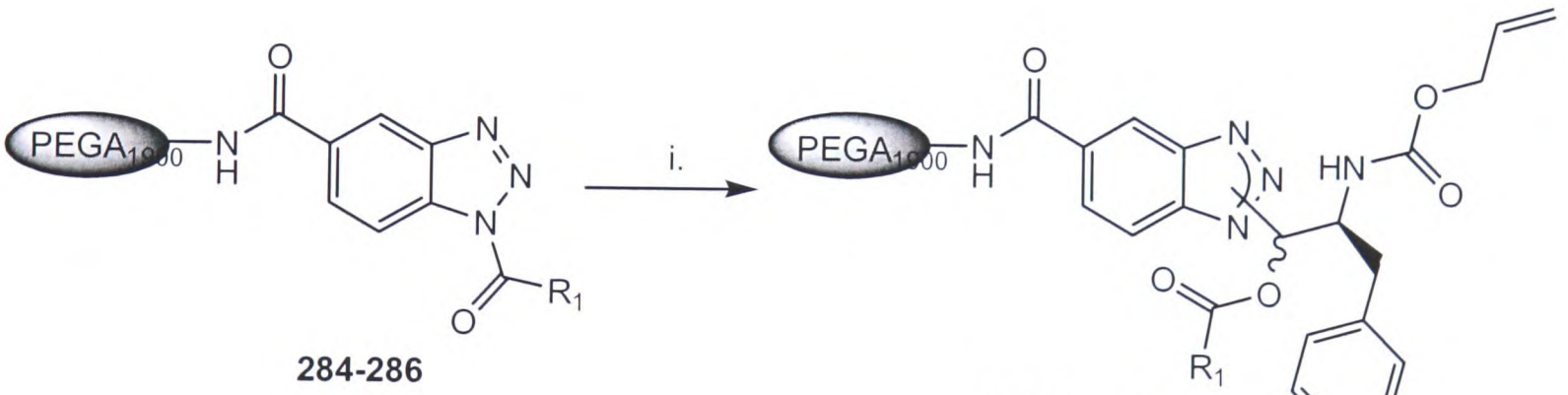
From Table 4, it can be seen that there is no obvious correlation between the molecular weight of the enzyme and the cleavage yield obtained. For example, for substrate **288**, Hog pancreas lipase (50kDa) gives a greater cleavage yield (42%) than *Mucor meihei* lipase (25kDa; 28%).

Enzyme	MWt of enzyme (kDa)	Cleavage (%)		
		288	289	290
Control	N/A	0	0	0
Candida antarctica lipase (CA)	35/45	15	-	-
Mucor Meihei lipase (MM)	25	28	1	-
Hog Pancreas lipase (PPL)	50	42	5	0
Candida Cylindracea lipase (CCL)	67	7	-	-
Mucor javanicus lipase (MJ)	21	7	-	-

Table 4: *Results of enzymatic cleavage of substrates* ***288-290****.*

6.2.6 Solid phase synthesis of diastereomeric hemiaminal linkers on PEGA$_{1900}$ resin

$PEGA_{1900}$-bound linkers **295-297** were synthesized using methodology developed previously (Section 6.2.4) (Scheme 82). For all resins, **295-297** evidence was found for the presence of the allyoxycarbonyl protecting group in the MAS ^1H NMR spectra.



Scheme 82: *Reagents and conditions:* i. (*S*)-**171**, Et_3N, acetonitrile, 20 h, r.t.

6.2.7 Enzymatic hydrolysis of PEGA$_{1900}$-bound hemiaminal linkers

Small scale hydrolysis reactions were carried out (500 mg of resin) using various lipases, an esterase and penicillin acylase. Each substrate **295-297** was treated with the appropriate cleavage mixture (Table 5) for 16 hours and the amount

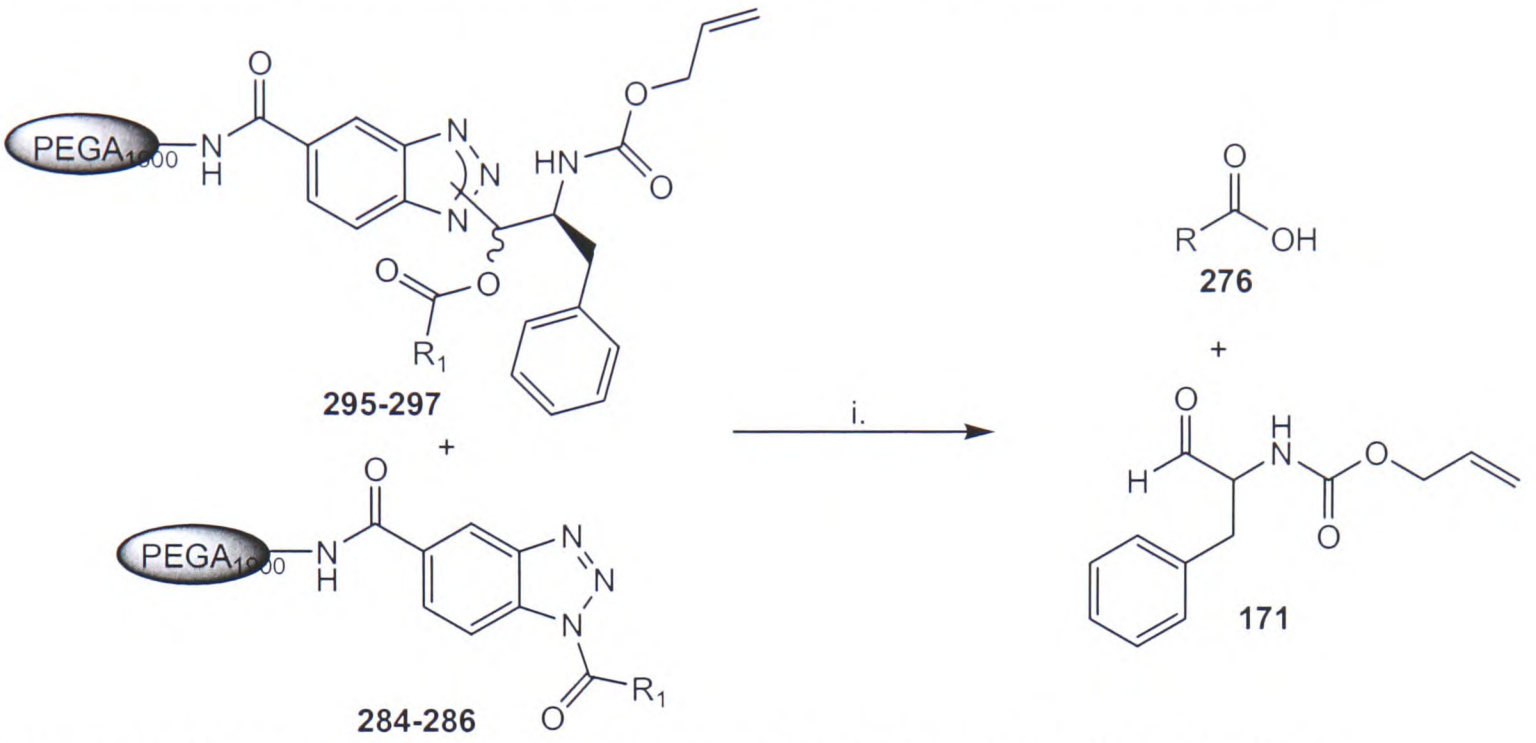
of corresponding acid released was quantified by reverse phase HPLC (Section 8.2.9).

Cleavage method	**Conversion (%)**		
	295	**296**	**297**
Control (MeOH)	0	0	0
7M NH_3/MeOH	100	100	100
Control (pH 7.4 buffer/10% CH_3CN)	3	10	4
Thermoanaerobium brockii esterase	2	5	3
Pseudomonas fluorescens lipase	0	11	0
Hog Pancreas lipase	2	14	3
Chromobacterium viscosum lipase	1	11	2
Candida lipolytica lipase	2	5	2
Mucor javanicus lipase	2	18	0
Penicillium roqueforti lipase	1	14	2
Rhizopus arrhizus lipase	0	9	0
Penicillin acylase	0	31	0

Table 5: *Results of enzyme cleavages of* ***295-297****.*

The results shown in Table 5 show that only the phenylacetate linker **296** is a substrate for the enzymes, with Penicillin acylase showing the best cleavage yields (31%) followed by *Mucor javanicus* lipase (18%). All substrates were re-treated with the corresponding cleavage mixture, however no further cleavage was observed. Further treatment of the substrates previously treated with an enzyme, with methanolic ammonia resulted in cleavage of the remaining aldehyde (*S*)-**171**. In all substrates, **295-297** small amounts of cleavage were observed in the acetonitrile/buffer control (3, 10 and 4% respectively). In all cases, **295-297** the appropriate breakdown figures (3, 10 and 4% respectively) were subtracted from the cleavage yields to give the actual cleavage yields for **295-297**. Allyloxycarbonyl-phenylalaninal **171/173** and corresponding acid **276** were observed in a ratio of 1:1.4 respectively in the reverse phase HPLC traces of all substrates **295-297**, as calculated by external standards. 70% of cleavage can be attributed to breakdown of the hemiaminal **295-297**, while 30% was generated from the *N*-acyl benzotriazoles **284-**

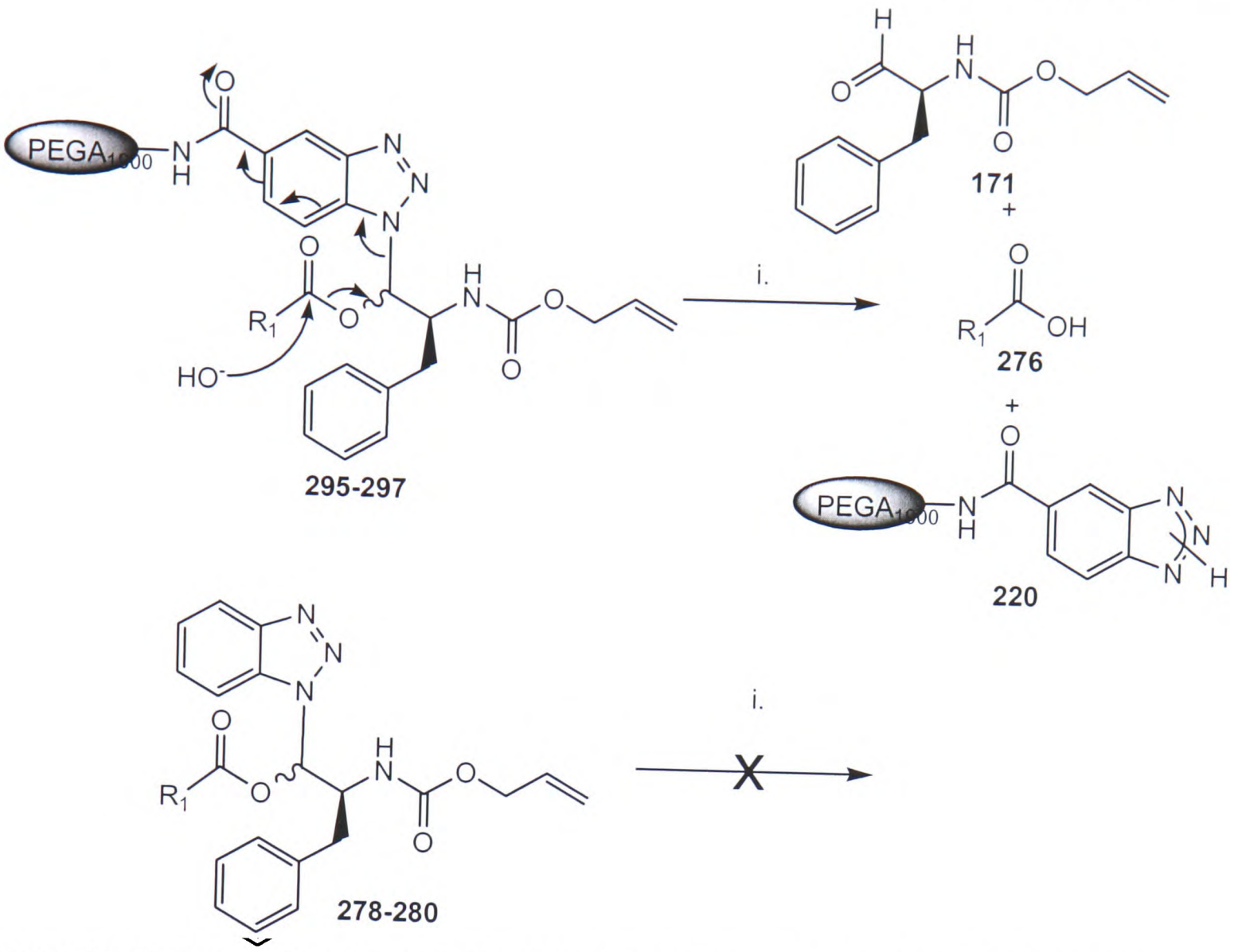
286. Presumably, the *N*-acyl benzotriazoles **284-286** were present due to incomplete coupling and these are known to be unstable in solution (Scheme 83). Reducing the cleavage time (2 hours) did not reduce the amount of breakdown observed; however, the amount of enzyme cleavage was substantially reduced (only 0-5% cleavage).



Scheme 83: *Reagents and conditions:* i. 10% acetonitrile/phosphate buffer pH 7.4, 2-16 h, r.t.

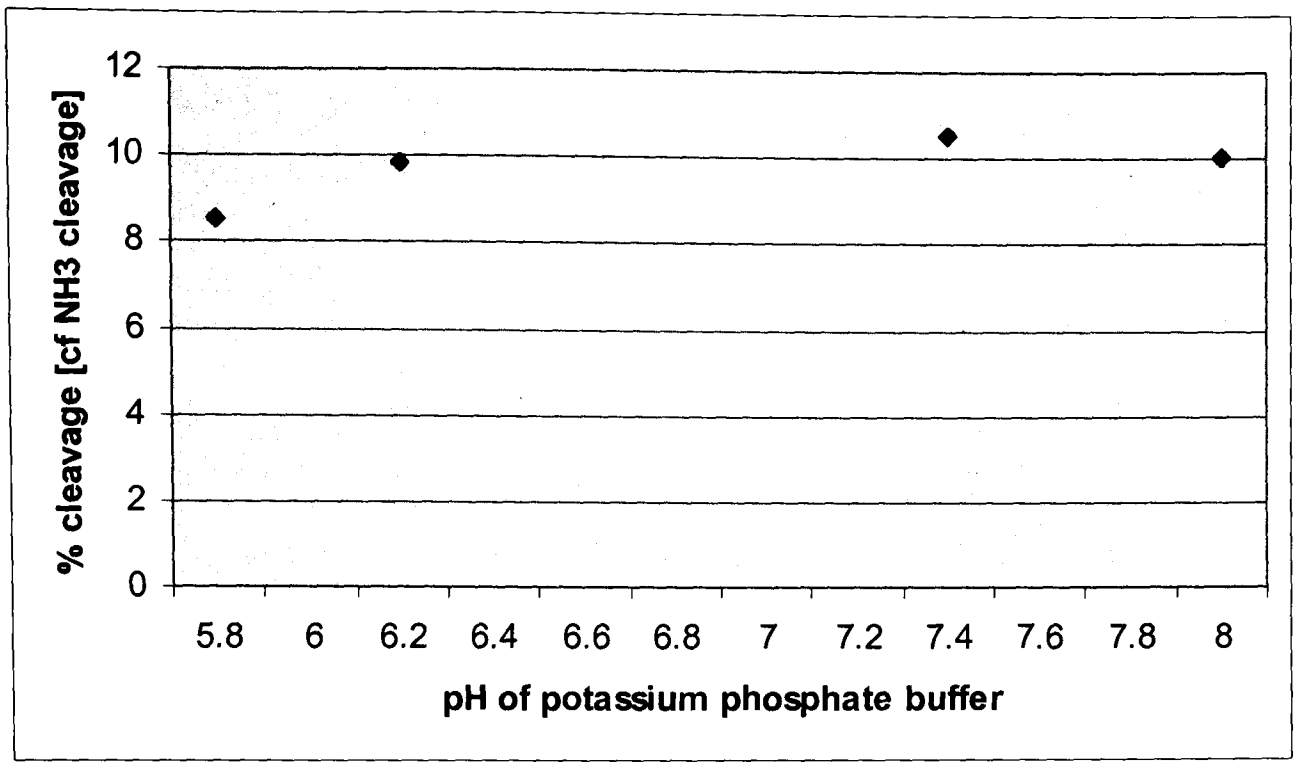
From the solution phase transformations of **278-280,** it can be seen that the lipases/esterase exhibit some diastereoselectivity. Therefore, it was reasonable to assume that the solid supported lipase/esterase hydrolysis would also show some diastereoselectivity; hence, it should be noted that the lipase/esterase hydrolysis of **295-297** might be limited to <50% conversion. Since this was impossible to determine, the diastereoselectivity of the solid phase enzymatic transformations, the yields were stated out of a theoretical 100% conversion.

Linkers **295-297** were stable in methanol since no acid **276** or aldehyde **171/173** was observed in the methanol control. In contrast to the behaviour of **295-297,** the corresponding solution phase analogues **278-280** were stable in buffer/acetonitrile solution; possibly because they do not possess the weakly electron withdrawing amide moiety (Scheme 84).



Scheme 84: *Reagents and conditions:* i. 10% acetonitrile/potassium phosphate buffer 10 mM, 16 h, r.t.

It has been reported[114] that lowering the pH can reduce the contribution from non-enzymatic hydroxide-induced hydrolysis (Scheme 84). Therefore, a study was carried out to determine whether the stability of the linker in buffer could be improved by carrying out the reaction in buffers of lower pH. Resin **296**, was treated with phosphate buffer (50 mM) at a variety of different pH values. The amount of phenylacetic acid released was quantified by reverse phase HPLC. The results shown in Graph 2, show that the largest cleavage was observed for buffer pH 7.4 (10.5%), however unacceptable cleavage yields were obtained for all other pHs tested (8.5-10%). It should be noted, that the linker **296**, was stable in water and methanol. To conclude, no significant pH dependence was observed for the stability of the phenylacetate linker **296**.



Graph 2: *pH dependence of* ***296*** *cleavage*

In order to determine the enantiomeric excess (e.e.) of the allyloxycarbonyl-phenylalaninal **171** released during enzymatic cleavage of **296**, **171/173** was reduced to the allyloxycarbonyl-phenylalaninol **174/175** using sodium borohydride. Unfortunately, the determination of the e.e. of (*S*)-allyloxycarbonyl-phenylalaninal **171** was hampered by the poor cleavage yields and lack of resolution of the chiral HPLC method. Eventually, a gradient elution method was developed that separated the enantiomers of the racemic allyloxycarbonyl-phenylalaninol **175** standard Figure 31. Only resin **296** cleaved by *Mucor javanicus* lipase and penicillin acylase provided enough alcohol **171/174** for analysis by chiral HPLC. Figure 32 and Figure 33 show the chiral HPLC traces of the *Mucor javanicus* lipase and penicillin acylase cleavage mixtures. It can be seen that both enzymes cleave the linker to give (*S*)-allyloxycarbonyl-phenylalaninal **171** with an enantiomeric excess of >98%.

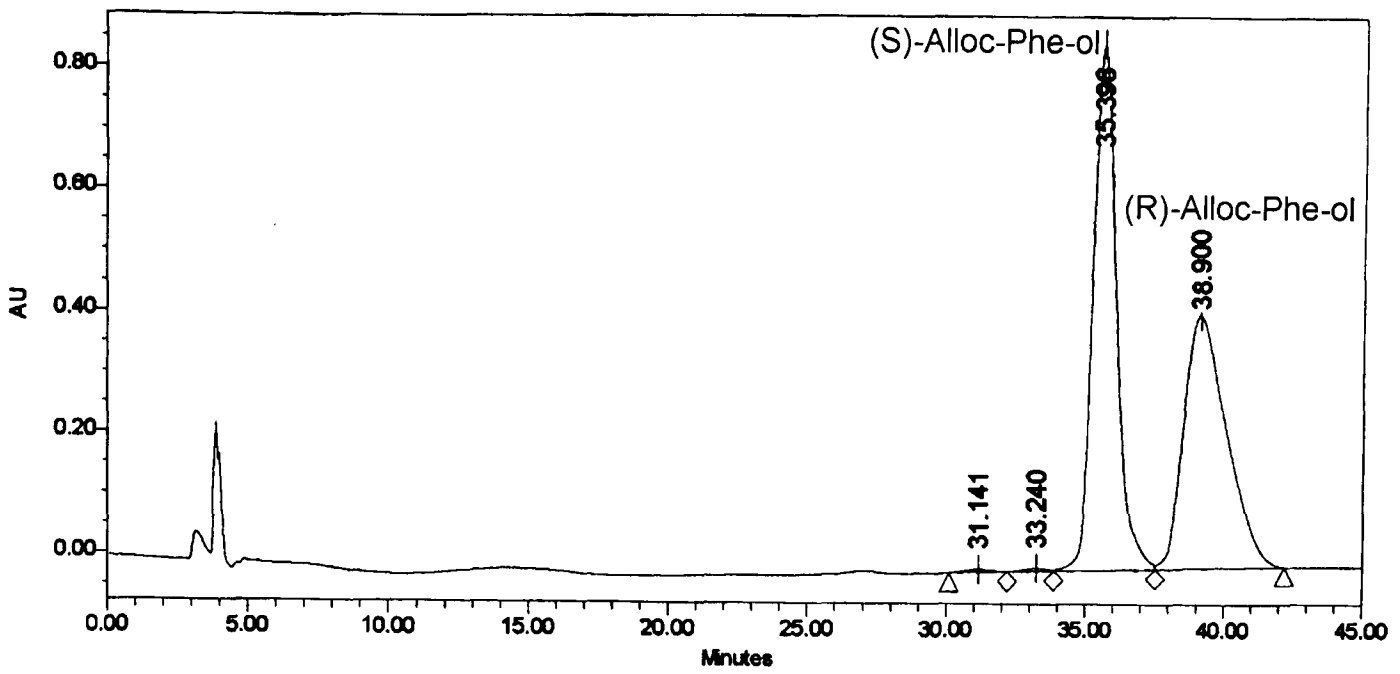


Figure 31: *Chiral HPLC trace of racemic allyloxycarbonyl-phenylalaninol* ***175.***

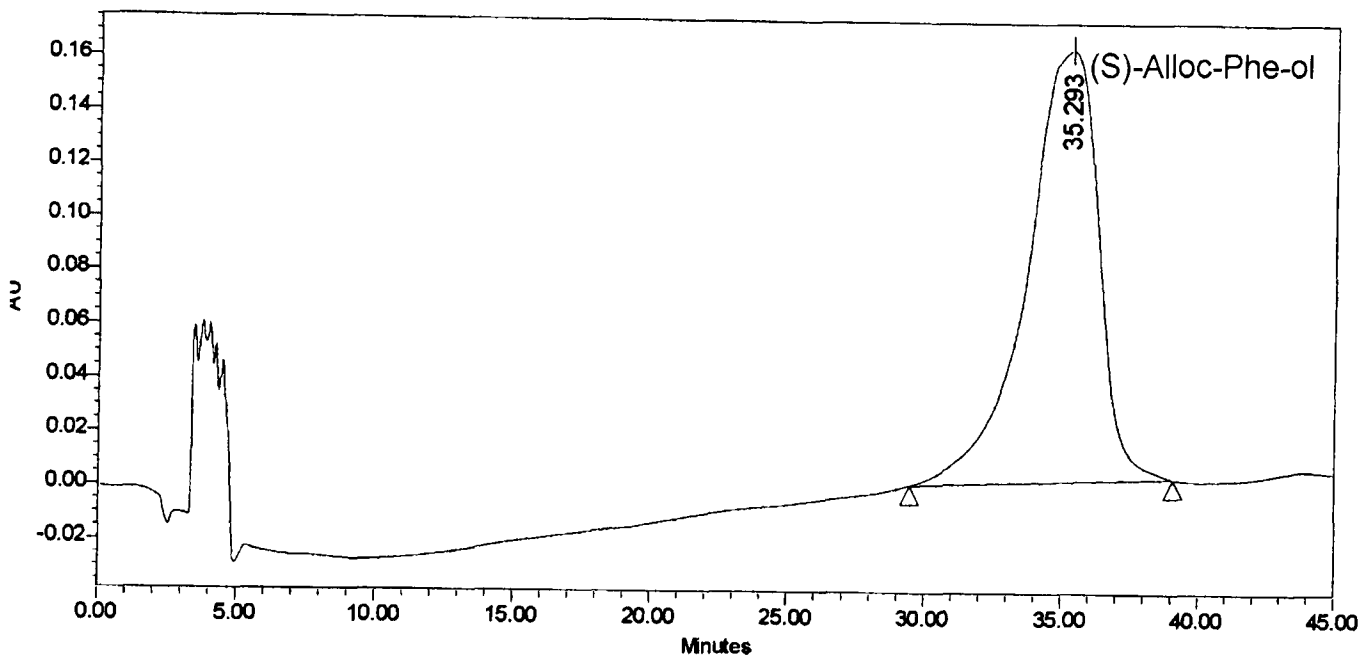


Figure 32: *Chiral HPLC trace of* ***296*** *cleavage by Mucor javanicus lipase.*

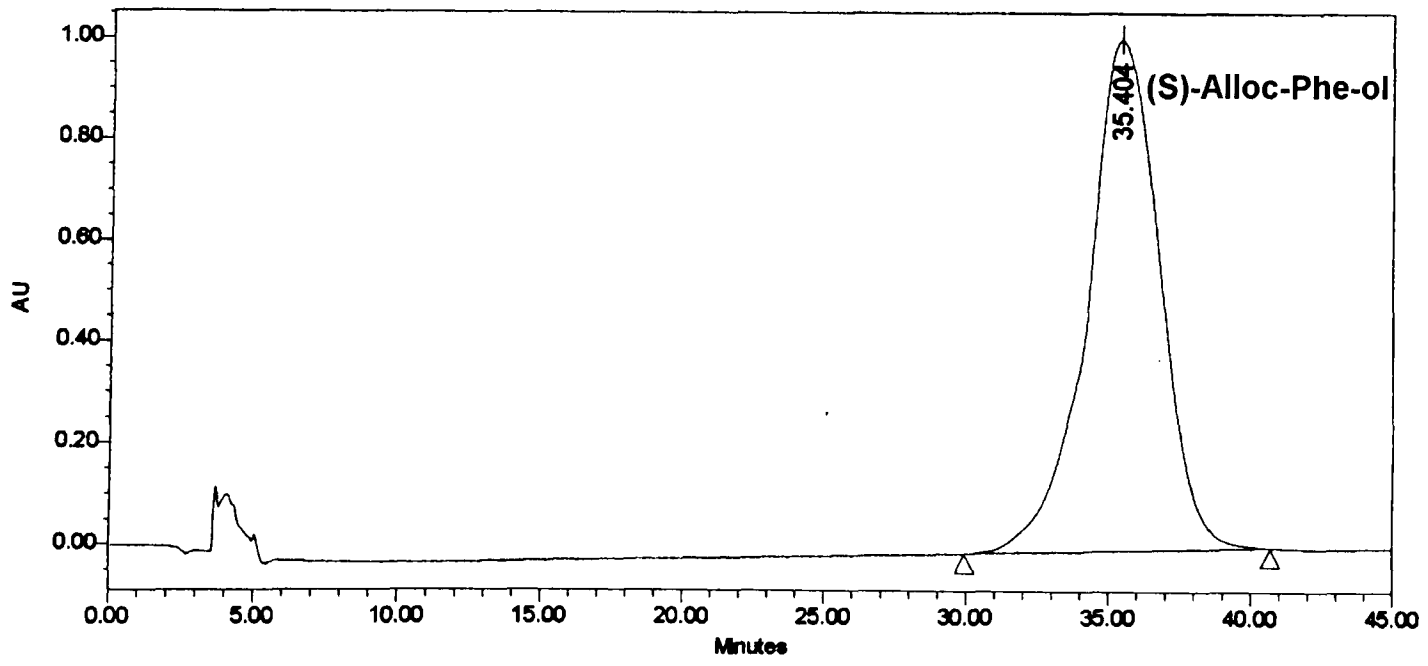
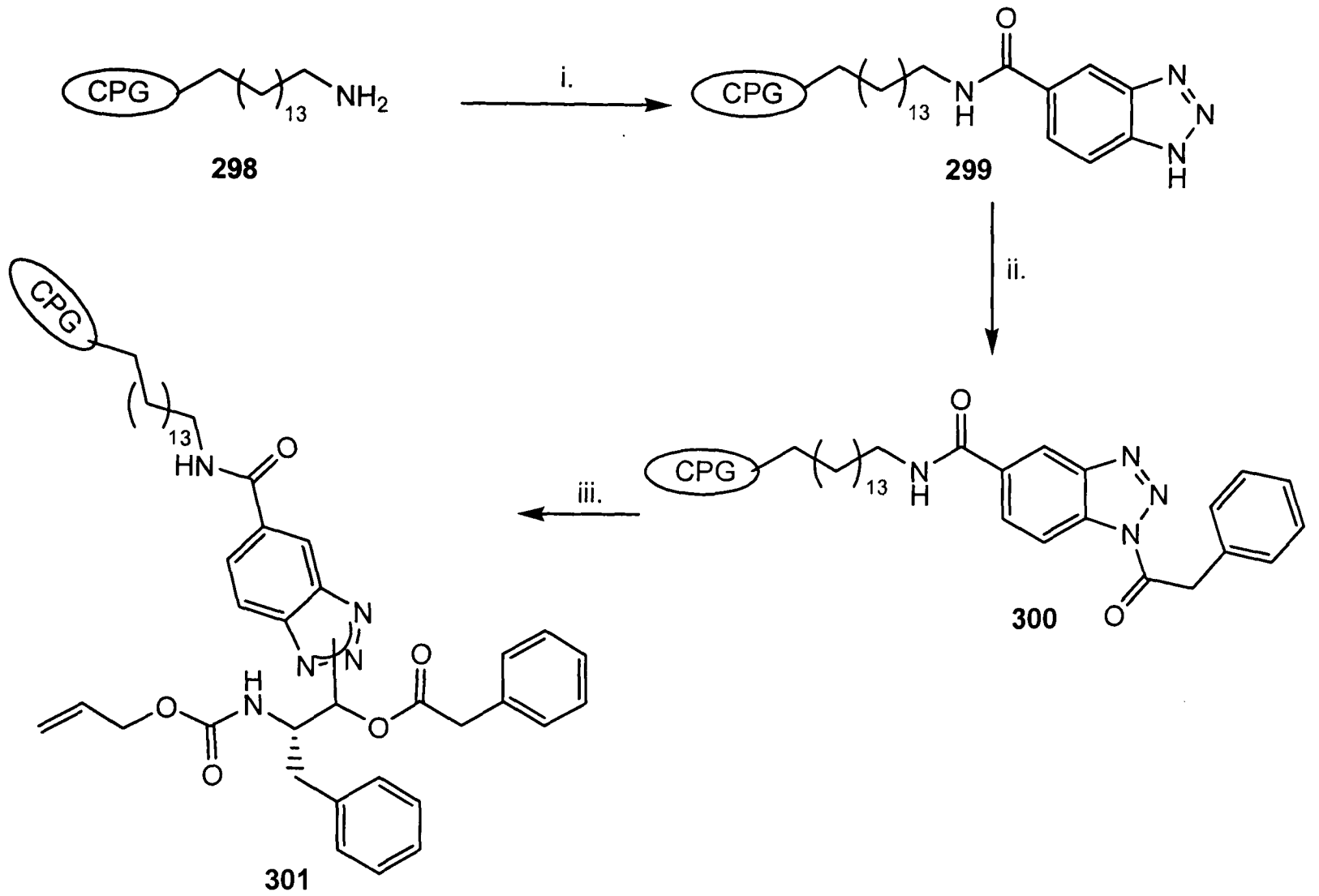


Figure 33: *Chiral HPLC trace of* ***296*** *cleavage by penicillin acylase.*

As mentioned previously (Section 1.2.5), CPG has been successfully utilised for enzymatic transformations; however, the pore size of CPG (*i.e.* 100 nm) is crucial for the accessibility of enzymes. With this in mind, CPG (pore size; 100 nm) was utilized as a support for the hemiaminal linker **301** and subsequent enzymatic cleavage was carried out. Initially, benzotriazole was coupled to aminopropyl-CPG (80.3 μmol g^{-1}, pore size; 101 nm) using diisopropylcarbodiimide and HOBt. Unfortunately, these reagents were unsuitable for the formation of **299**. Optimisation of the coupling conditions led to the use of HBTU and HOBt for the formation of **299** (73 μmol g^{-1}, 92% loading from *N* analysis). The propyl spacer only allows a short distance between the silica backbone and the reactive amine; therefore, it was thought that the silica backbone could sterically hinder the amide coupling. This theory led us to postulate that by using a longer alkyl spacer, the contribution from steric hinderance could be minimized and penetration of the linker into the enzyme could be maximized. Long-chain alkyl-amine CPG **301** (15 carbon extension arm, pore size; 108 nm) was purchased from CPG Inc (now Millipore Ltd) and coupled to benzotriazole 5-carboxylic acid **207** to give **299** with moderate loading yields (63% from *N* analysis) (Scheme 85). The decrease in loading yields can be attributed to the high flexibility of the 15-carbon extension arm compared to the relatively inflexible propyl alkyl spacer arm. Time restraints prevented optimization of this yield; however, longer reaction times may provide better loading yields. The controlled pore glass bound benzotriazole **298** was characterized by FT-IR. The MAS ^{1}H NMR of resin **299,** was unattainable for the same reason as the macroporous-polystyrene bound benzotriazole **219** (Scheme 55). Additionally, gel-phase ^{13}C NMR spectra of CPG resin **299**, was impossible due to the non-swelling properties of the CPG resin. The acetal linker **301** was constructed using the method developed previously (Scheme 85) with an overall loading of 40.0 μmol g^{-1} (53%).

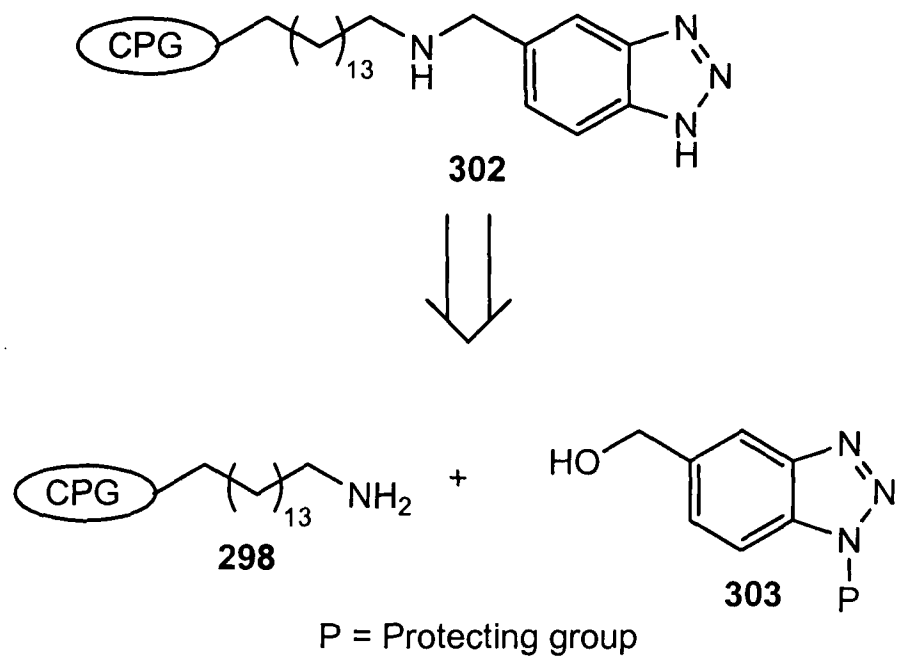


Scheme 85: *Reagents and condtions:* i. HBTU, HOBt, DIPEA, benzotriazole 5-carboxylic acid, DMF, 16 h, r.t.; ii. phenylacetyl chloride, Et_3N, anhydrous DCM, 20 h, r.t.; iii. (*S*)-**171**, Et_3N, acetonitrile, 20 h, r.t.

Enzyme hydrolysis experiments were carried out using *Mucor javanicus* lipase and penicillin acylase as before (Section 6.2.5). These enzymes were chosen due to their moderate success with $PEGA_{1900}$ resin. Unfortunately, it was found that no enzymatic hydrolysis of **301** was observed for either of the enzymes tested. One possible reason for this could be that the hydrophobic 15-carbon spacer is too flexible or may aggregate under the aqueous conditions required for enzymes. Therefore, the use of a more hydrophilic or rigid spacer may improve the enzyme cleavage.

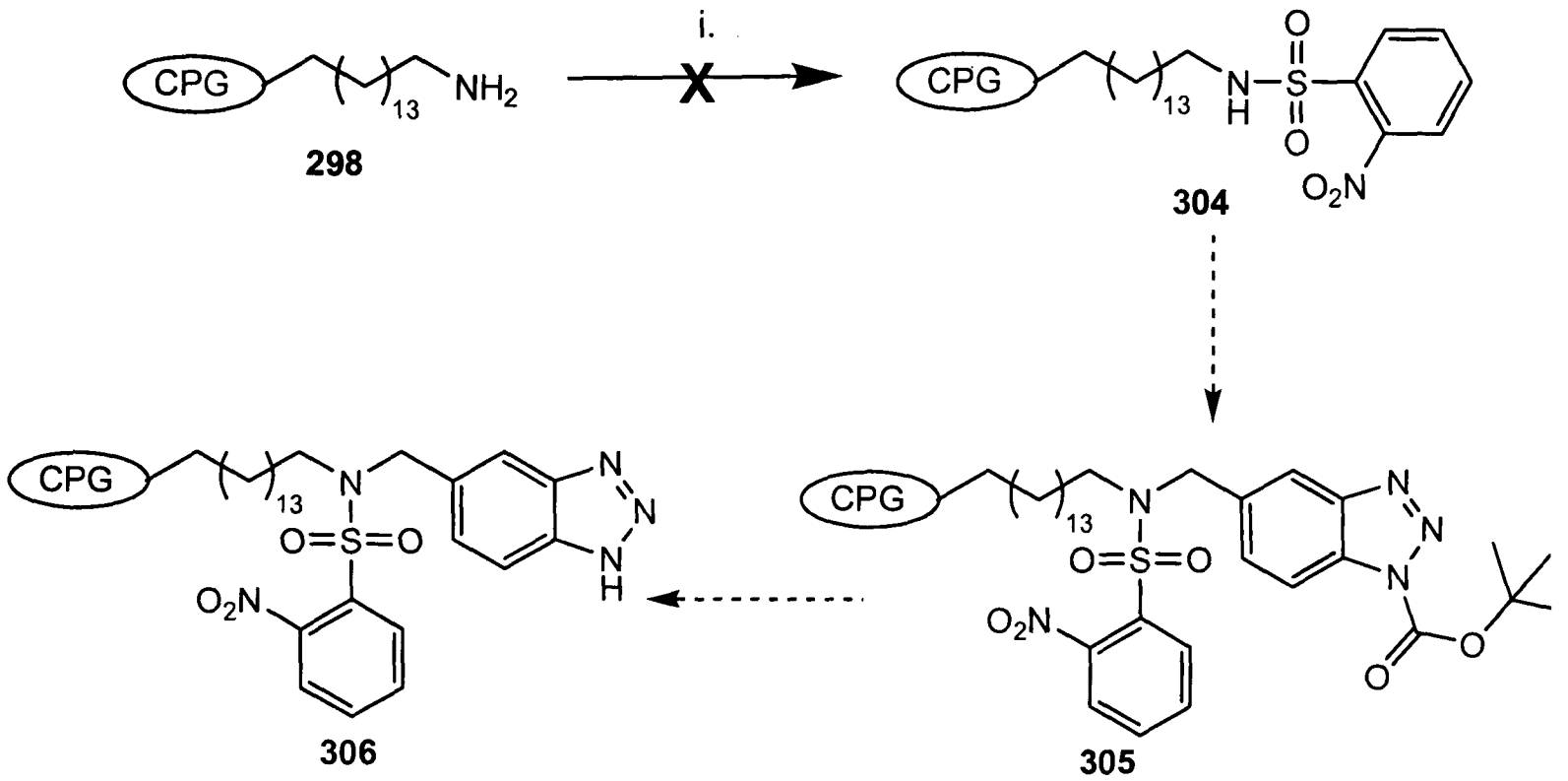
It was proposed that if benzotriazole was bound to the resin using an alkyl amine **302** (Scheme 86), the linker may be stable in phosphate buffer pH 7.4. The formation of the secondary amine **302** was envisaged using Mitsunobu chemistry.[115] Generally, this procedure requires that the primary amine **298** is converted into a derivative in which the NH is strongly acidic. This can be achieved through the formation of toluenesulfonamide,[116] trifluoroacetamide,[117] trifluoromethane-

sulfonamide,[118] 2,2,5,7,8-pentamethylchroman-6-sulfonyl (pmc)[119] and 2-nitrobenzenesulfonamide (Nbs)[120] derivatives. The method of choice for the linker synthesis was the 2-nitrobenzenesulfonamide (Nbs) derivative since this can be deprotected under neutral conditions using sodium thiophenolate in DMF.[121]



Scheme 86: *Retrosynthetic analysis of alkyl amine linked benzotriazole **302**.*

The synthesis of Nbs-protected amine **304** was attempted using 2-nitrobenzene sulfonyl chloride in DMAP as base (Scheme 87); however, this coupling was unsuccessful even after 3 repeated couplings for 1.5 h, 3h and 16h, at room temperature. This was presumably due to the 2-nitrobenzene sulfonyl chloride being unstable under such conditions. Yang and Chiu[121] reported optimum conditions for this coupling, using 2-nitrobenzene sulfonyl chloride and diisopropylethylamine in THF for 3 h. Unfortunately, these conditions were also unsuccessful in forming **304** and no further experiments were performed.



Scheme 87: *Attempted synthesis of Nbs-protected amine; Reagents and conditions;* i. 2-nitrobenzene sulfonyl chloride (4eq.), DMAP (6eq.), THF/DCM (2:1).

6.2.8 Molecular modelling

Molecular modelling was performed in an attempt to explain the low yields obtained for the penicillin acylase cleavage of allyloxycarbonyl-phenylalaninal **171** from solid support **296** (31%) and in solution **279** (27%).

The crystal structure of penicillin G acylase was obtained from the Protein Data Bank[122,123] and the structure prepared for energy minimization using the CHARMm forcefield with InsightII on a Silicon Graphics workstation. The water and penicillin G were separated from the protein and an assembly was created. The amino acid residues, Glu1 and Gln2 were missing from the α-subunit. Since, these residues were situated at the termini of the α-subunit, it was decided that these were unimportant in the binding of the substrate within the active site. Therefore, these residues were omitted from the penicillin acylase structure. The bond order was turned on and hydrogen atoms were added to the crystal structure. Potentials and partial charges were assigned and the energy minimization carried out on the modified enzyme.

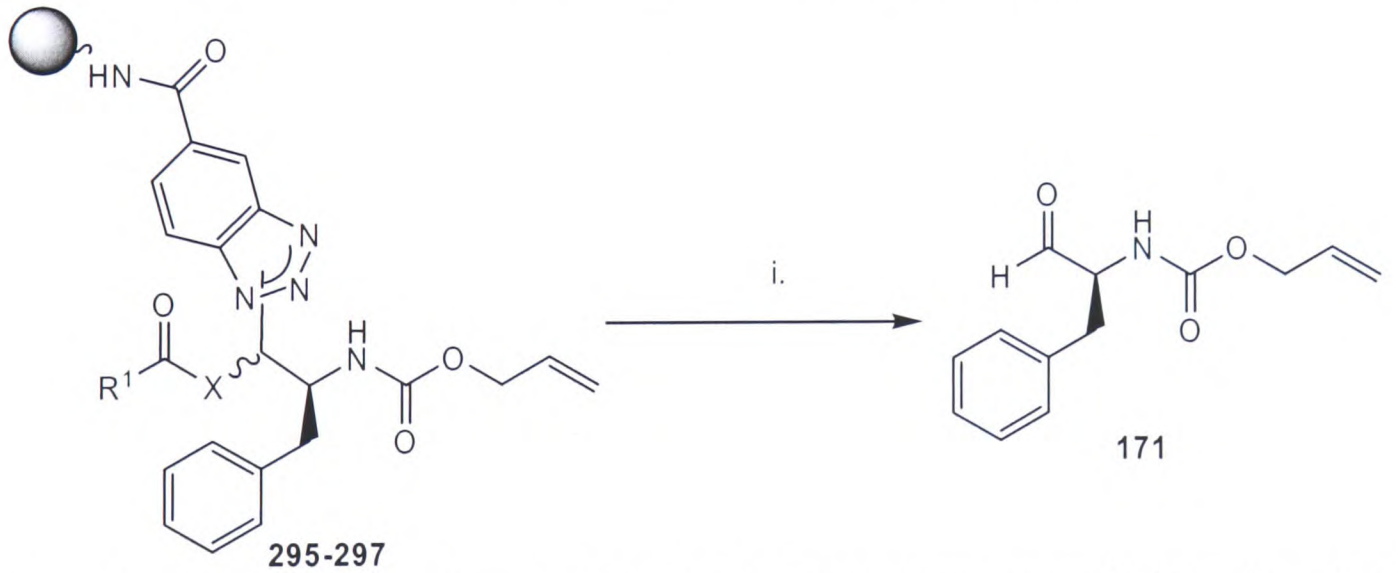
One stereoisomer of the linker, (*R,S*)-**296** was constructed in 2D, converted to 3D and energy minimization calculations were carried out to obtain the approximate energy minimum configuration. Manual docking was performed to position the linker within the binding site of the enzyme. Models of the linker **296**/penicillin acylase complex were compared to penicillin G bound to the active site of penicillin acylase (Appendix B and C). On examination of the penicillin G/enzyme complex, it could be seen that favourable aromatic interactions exist between the phenylacetamide of the penicillin G and the enzyme phenylalanine β24 and α146 residues. In addition, phenylalanine β57 is situated at the bottom of the hydrophobic cleft and although the distance between the substrate and this residue is too long for a direct interaction (4.9 Å). This residue may be important for maintaining the structure of the binding site. From Appendix B and C it can be seen that phenylalanine β71 has a favourable interaction with the hydrophobic β-lactam nucleus. The phenylalanine β24 and α146 residue interactions are shared in the molecular models of the linker **296**/enzyme complex (Appendix D-G). It appeared that the phenyl moiety fits into the hydrophobic pocket which was lined with many hydrophobic side chains such as isoleucine β177, alanine β69 and proline β22. In addition, phenylalanine β71 seemed to have a favourable hydrophobic interaction with the benzyl group on the linker. From the space filled and Connelly electron-density models (Appendix G and D), it seemed that the (*R,S*)-stereoisomer of the linker **296** would fit in the active site of the enzyme. On the other hand, it seems that the active site would be much more crowded with linker **296** bound to the active site, compared to Penicillin G in the active site (Appendix B). Examination of the two diastereoisomers of the linker **296** (Appendix H) showed that the position of the benzyl and Alloc groups in the (*S,S*)-**296** would be reversed compared to the (*R,S*)-**296**. In this case, the benzyl substituent would point into the hydrophobic cleft and the Alloc group would be positioned away from the hydrophobic cleft. In this position the benzyl group would still favourably interact with the phenylalanine β71 within the hydrophobic cleft. This suggested that both diastereomers of **296** were likely to be suitable for penicillin acylase hydrolysis. This is in agreement with the results of the solution phase study of benzotriazole hemiaminal **279**, hydrolysis by penicillin acylase, which showed no preference for either diastereomer (Table 2). It

was difficult to predict whether the solid support bound linker would fit in the active site, since the solid support cannot be modelled to any degree of accuracy; therefore, the influence the solid support would have on the active site binding was impossible to determine.

To conclude, molecular modelling explained the limited success of the enzymatic cleavage of **279** and **295**. Although **279/295,** seemed to interact favourably with the active site; it seemed that steric hinderance between the linker **279/295** and penicillin acylase limited the enzymatic hydrolysis yields.

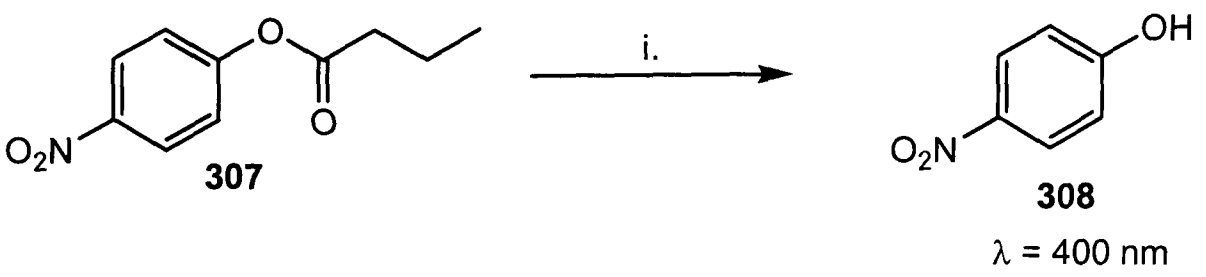
6.2.9 Lipase/Penicillin acylase inhibition assays

Since peptide aldehydes are known inhibitors of serine proteases, it seemed possible that the (*S*)-allyloxycarbonyl-phenylalaninal **171** cleaved during the hydrolysis, could inhibit the lipases/penicillin acylase (*i.e.* product inhibition) (Scheme 88) and limit the amount of enzymatic hydrolysis observed.



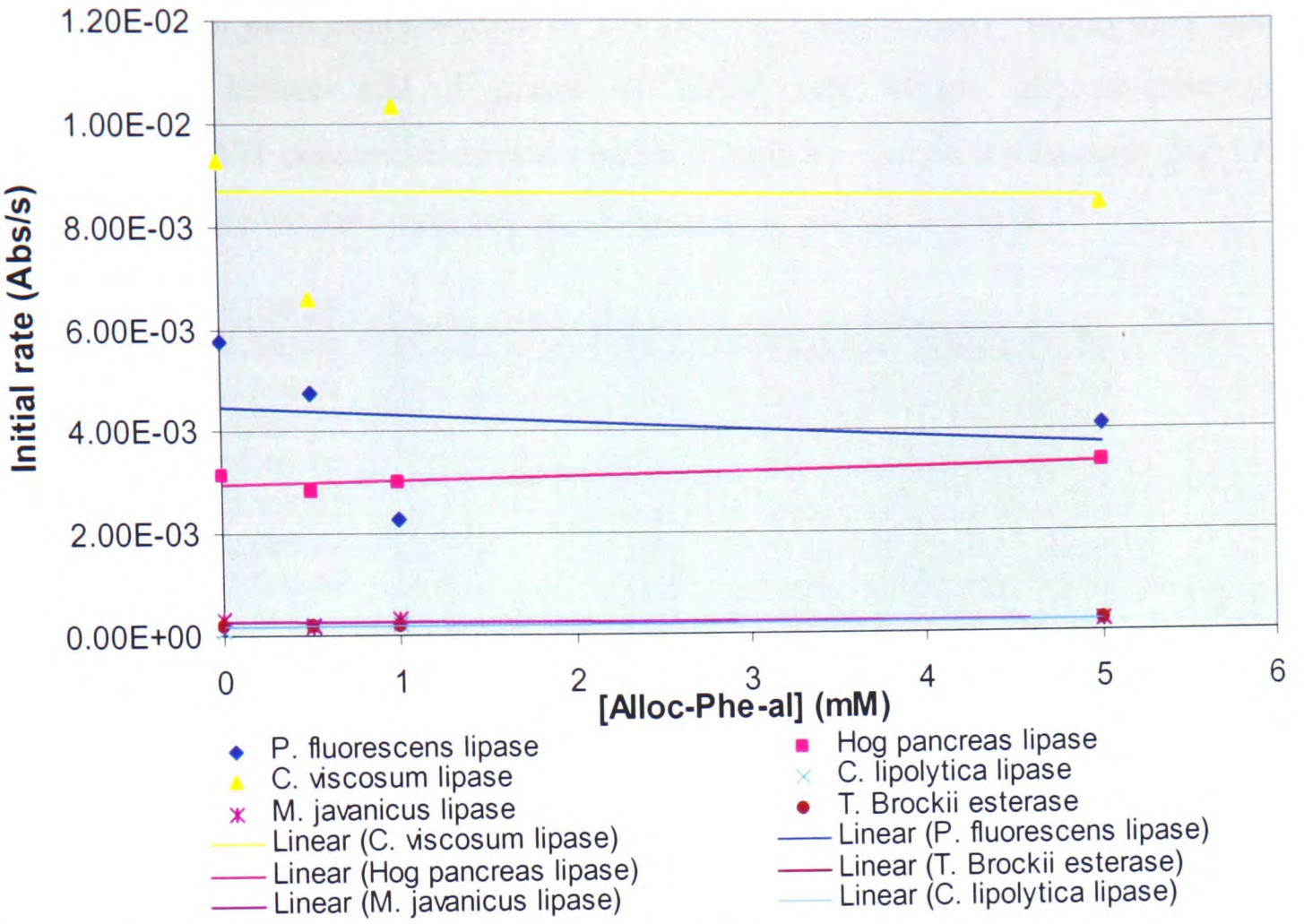
Scheme 88: *Reagents and conditions:* i. When X = O, lipase, esterase or penicillin acylase, pH 7.4 buffer; When X = NH, penicillin acylase, pH 7.4 buffer.

The hydrolysis of the carboxylic ester of *para*-nitrophenol **307** leads to the release of *p*-nitrophenol **308. 308** can be monitored continuously and quantitatively using a spectrophotometric method (Scheme 89). The appearance of the yellow coloured *para*-nitrophenol **308** (pK_a 7.15) can be monitored by reading the increase in absorbance at 400 nm[124,125].



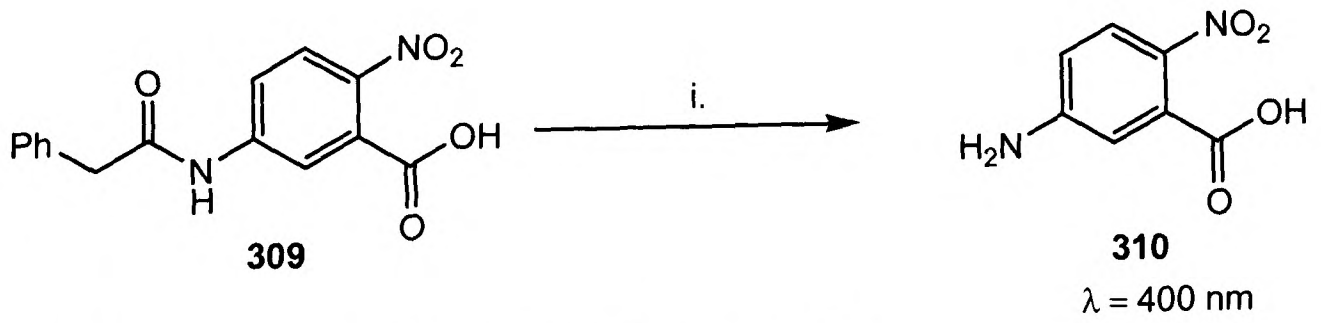
Scheme 89: *Reagents and conditions:* i. lipase or esterase, pH 7.4.

The reaction rate of the lipases/esterase hydrolysis of *para*-nitrophenylbutyrate **307** was determined using a method by Pencreac'h and Baratti[126]. For each enzyme, a suspension of *para*-nitrophenylbutyrate **307** (10 mM) (1 mL) in 10 mM phosphate buffer pH 7.4 was placed in a 3 mL cuvette. The desired concentration of allyloxycarbonyl-phenylalaninal **171** was added (150 μL) [0, 0.5, 1 and 5 mM], followed by 150 μL of stock enzyme suspension [(1 mg mL^{-1}) in 10 nM phosphate buffer pH 7.4] and the absorbance of the *para*-nitrophenol **308** was read at 400 nm against a blank without enzyme. This was repeated for each concentration of allyloxycarbonyl-phenylalaninal **171** [0, 0.5, 1 and 5 mM]. The maximum concentration of allyloxycarbonyl-phenylalaninal **171** (5 mM) was chosen so that the concentration of **171** in the assay exceeded the maximum concentration of **171** that could be cleaved from PEGA bound linker with a 0.2 mmol g^{-1} loading (i.e. 50 mg resin = 1 mM). Initial rates were calculated from the slope of the line of the data points obtained when plotting absorbance versus the time. Graph 3 shows a plot of initial rates versus allyloxycarbonyl-phenylalaninal **171** concentration. If **171** was an inhibitor, a significant decrease in initial rate should occur with increasing concentrations of **171**. In general, it can be seen that none of the lipases or the esterase are inhibited by the allyloxycarbonyl-phenylalaninal **171** to any considerable extent, at the concentrations tested (0-5 mM). It should be noted that, as the initial rates of the enzyme reactions increase the errors become larger. This could account for the large fluctuations in points for the two fastest acting enzymes, *Chromobacterium viscosum* and *Pseudomonas fluorescens* lipase.



Graph 3: *Initial rate vs. 171 concentration for lipase/esterase.*

A similar method was employed for monitoring the inhibition of penicillin acylase. The hydrolysis of 5-(2-phenylacetamido)-2-nitrobenzoic acid **309** with penicillin acylase results in the release of 5-amino-2-nitrobenzoic acid **310** which can be quantitatively measured using UV detection at 400 nm (Scheme 90).



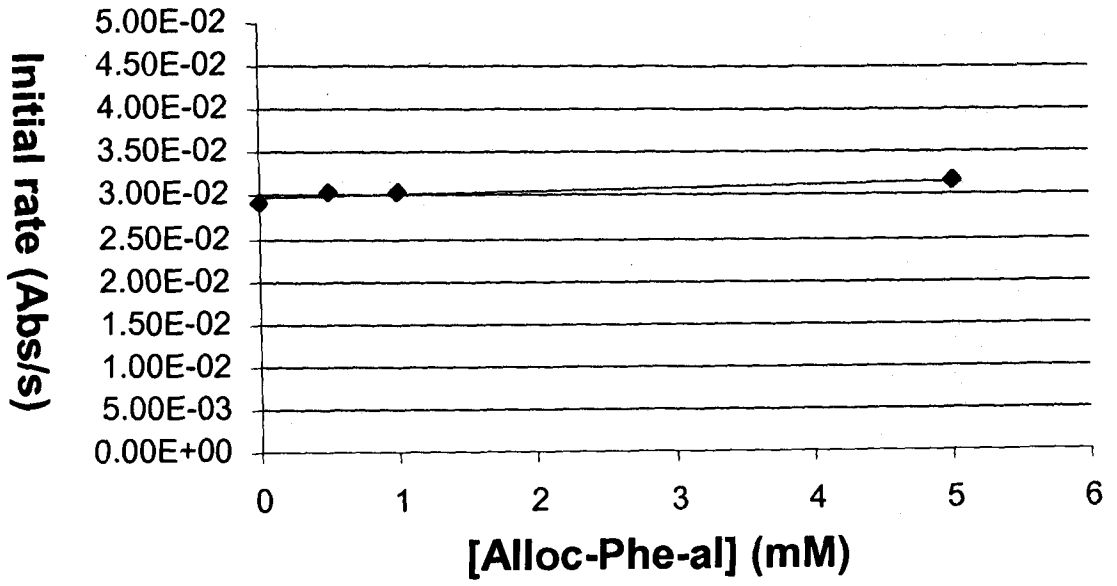
309 **310**

$\lambda = 400$ nm

Scheme 90: *Reagents and conditions:* i. Penicillin acylase, pH 7.4.

To a solution of 5-(2-phenylacetamido)-2-nitrobenzoic acid **309** in phosphate buffer pH 7.4 (1 mL) in a 3 mL cuvette, was added the required concentration of **171** (150 μL) (0, 0.5, 1 and 5 mM) in buffer, followed by penicillin acylase [(1 mg mL^{-1}) in 10 nM phosphate buffer pH 7.4] (150 μL). The absorbance of 5-amino-2-nitrobenzoic acid **310** was recorded at 400 nm against a blank without enzyme. This

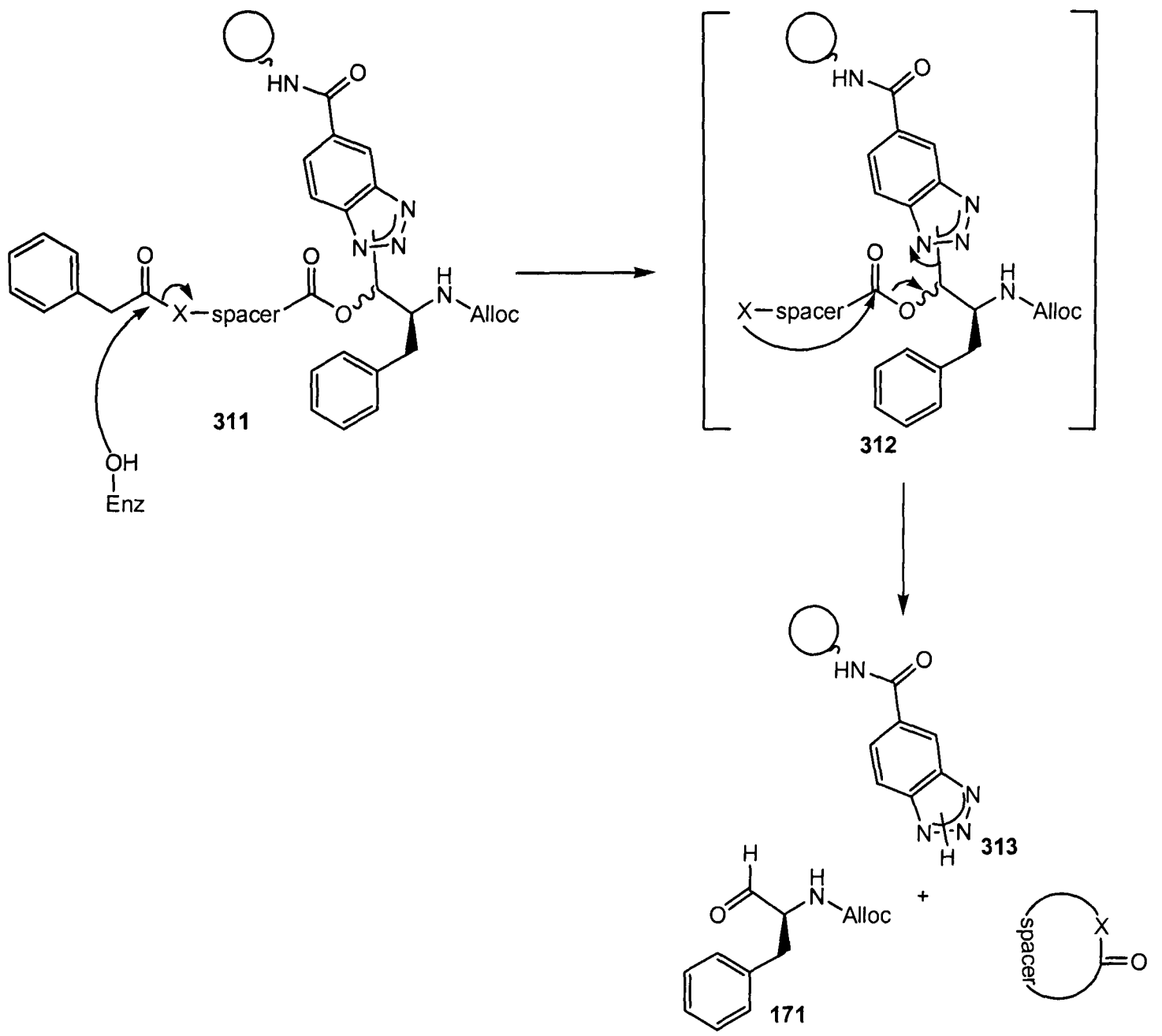
was repeated for each concentration of **171** (0, 0.5, 1 and 5 mM). Initial rates were calculated as before and a graph of initial rate versus allyloxycarbonyl-phenylalaninal **171** concentration was plotted (Graph 4). Graph 4 illustrates that **171** is not an inhibitor for penicillin acylase at concentrations up to 5 mM.



Graph 4: *Initial rate vs. 171 concentration for penicillin acylase*

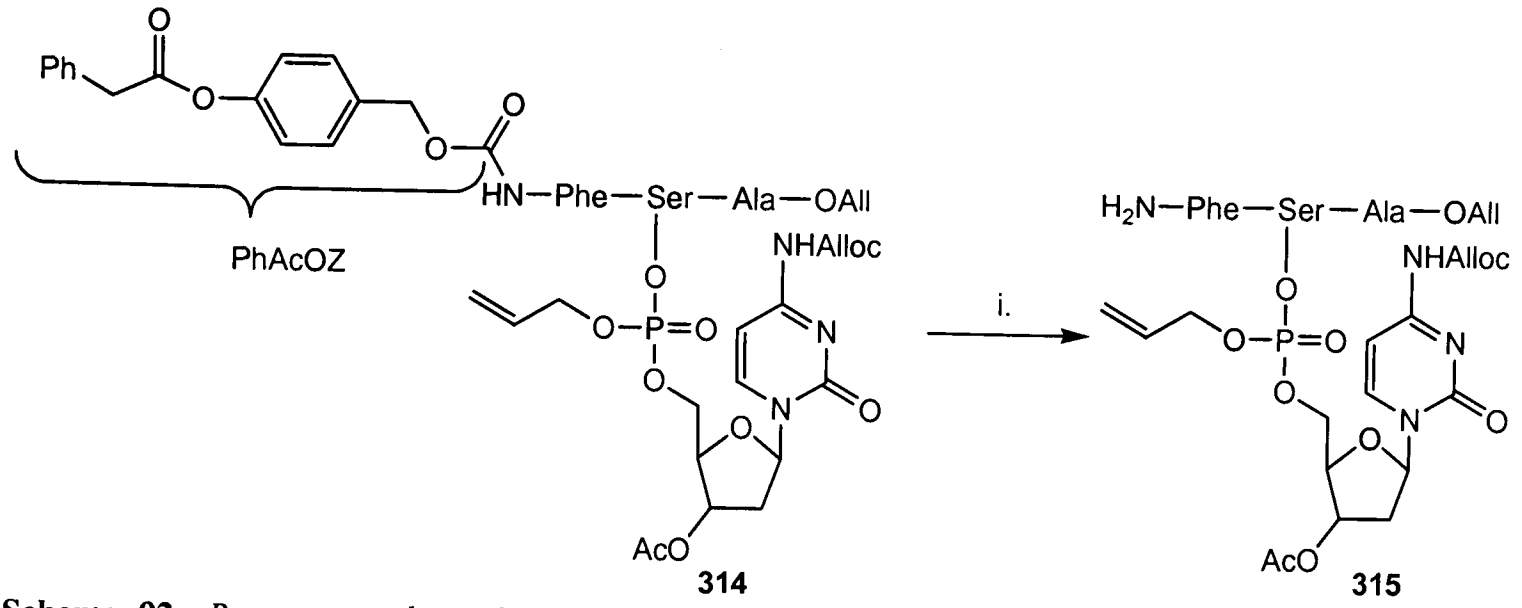
6.2.10 Solid phase synthesis of safety catch linker

Molecular modelling suggested that the enzymatic hydrolysis of the linkers **295-297**, may be hampered by unwanted steric or electronic interactions between the linker/substrate and the biocatalyst. Therefore, the substrate tolerance of the biocatalyst may be limited. The use of a spacer unit between the site of attack and the asymmetric carbon should maximize the substrate tolerance of the enzyme, hence possibly improve the enzymatic cleavage yields. From the proposed scheme (Scheme 91), it is obvious that the cleaved linker would need to fragment further to release the desired aldehyde **171**. Linkers of this type are known as 'Safety catch linkers'.



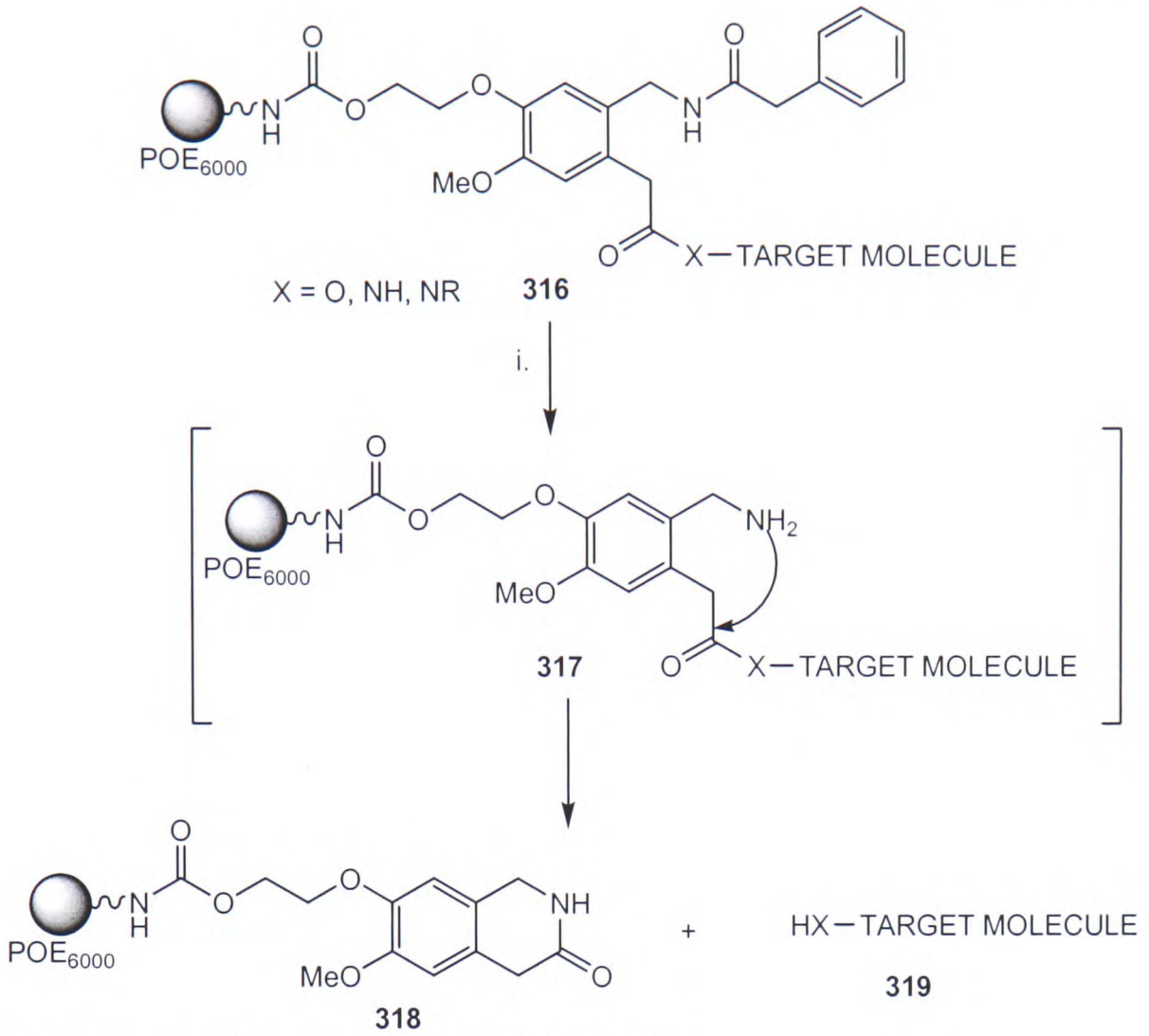
Scheme 91: *Proposed mechanism for release of aldehyde 171 from extended linker 311.*

In a report published in 2001, Waldmann *et. al.*[127] used an enzyme labile phenylacetoxy benzyloxycarbonyl (PhAcOZ) group for the *N*-protection of nucleotripeptides **315** (Scheme 92). Penicillin G acylase cleavage of the phenylacetyl group **314**, followed by spontaneous fragmentation released the nucleotripeptide **315** in 69% yield. It should be noted that only solution phase cleavage was reported. The high selectivity of penicillin G acylase for the phenylacetyl group guaranteed that the other sensitive functional groups remained intact.



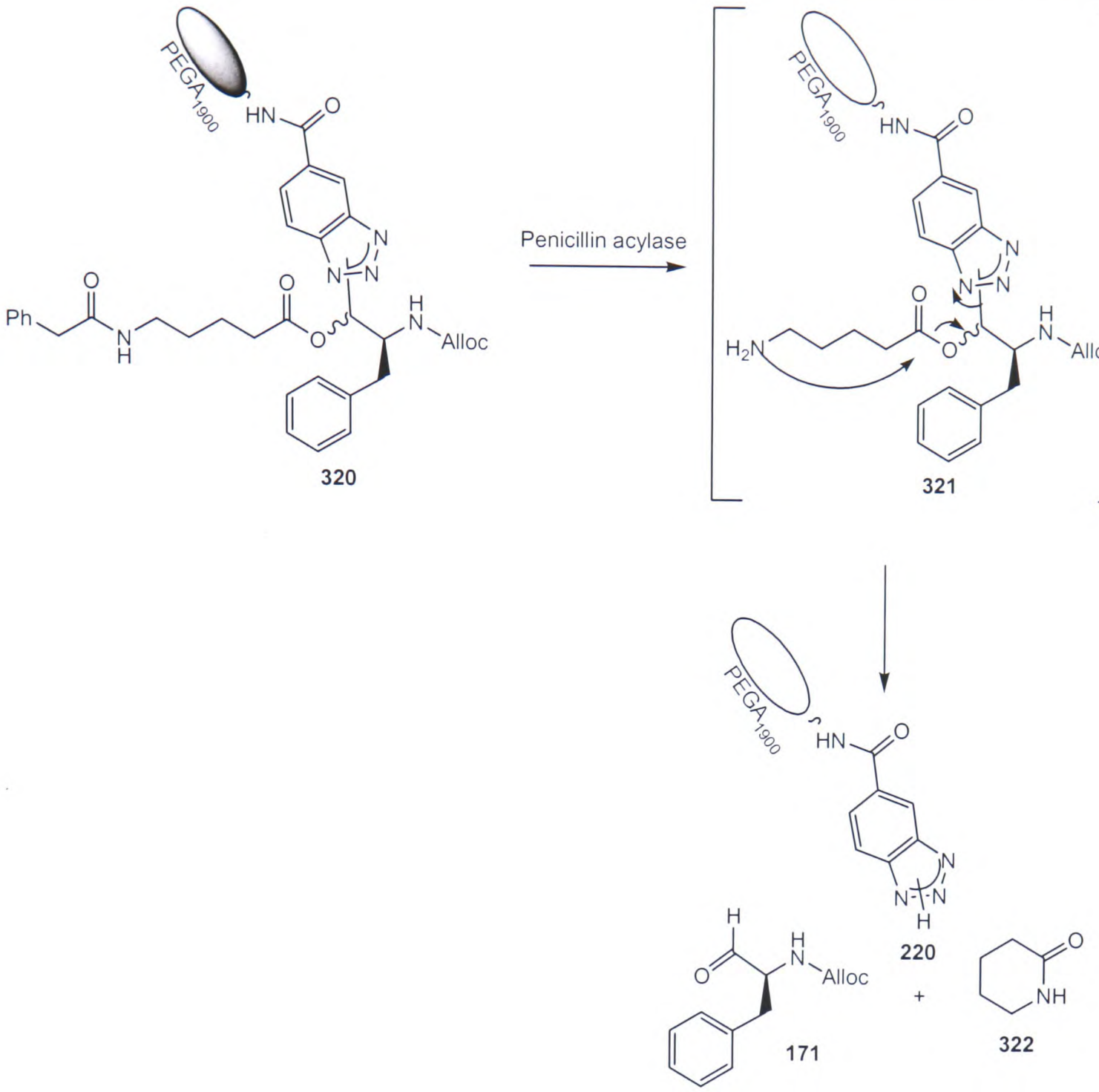
Scheme 92: *Reagents and conditions:* penicillin G acylase, 50 mM phosphate buffer, MeOH (20 vol%), 0.1 M KI, pH 6.8, 24 h, r.t., 69%.

In 2000 and 2001, Waldmann *et. al.*[43,44] reported an enzyme-labile safety catch linker **316** for synthesis on a soluble polymer support (Scheme 93). Penicillin acylase hydrolyses the phenylacetamide **316**, the benzylamine **317** cylises to polymer bound lactam **318** and releases the corresponding target molecule **319**. It is thought that the phenyl ring assists in the cyclization by bringing the reactive ends into closer proximity of each other, therefore reducing the entropy difference ($\Delta S^{\ddagger}$) and the enthalpy of activation becomes more favourable ($-\Delta G^{\ddagger}$).



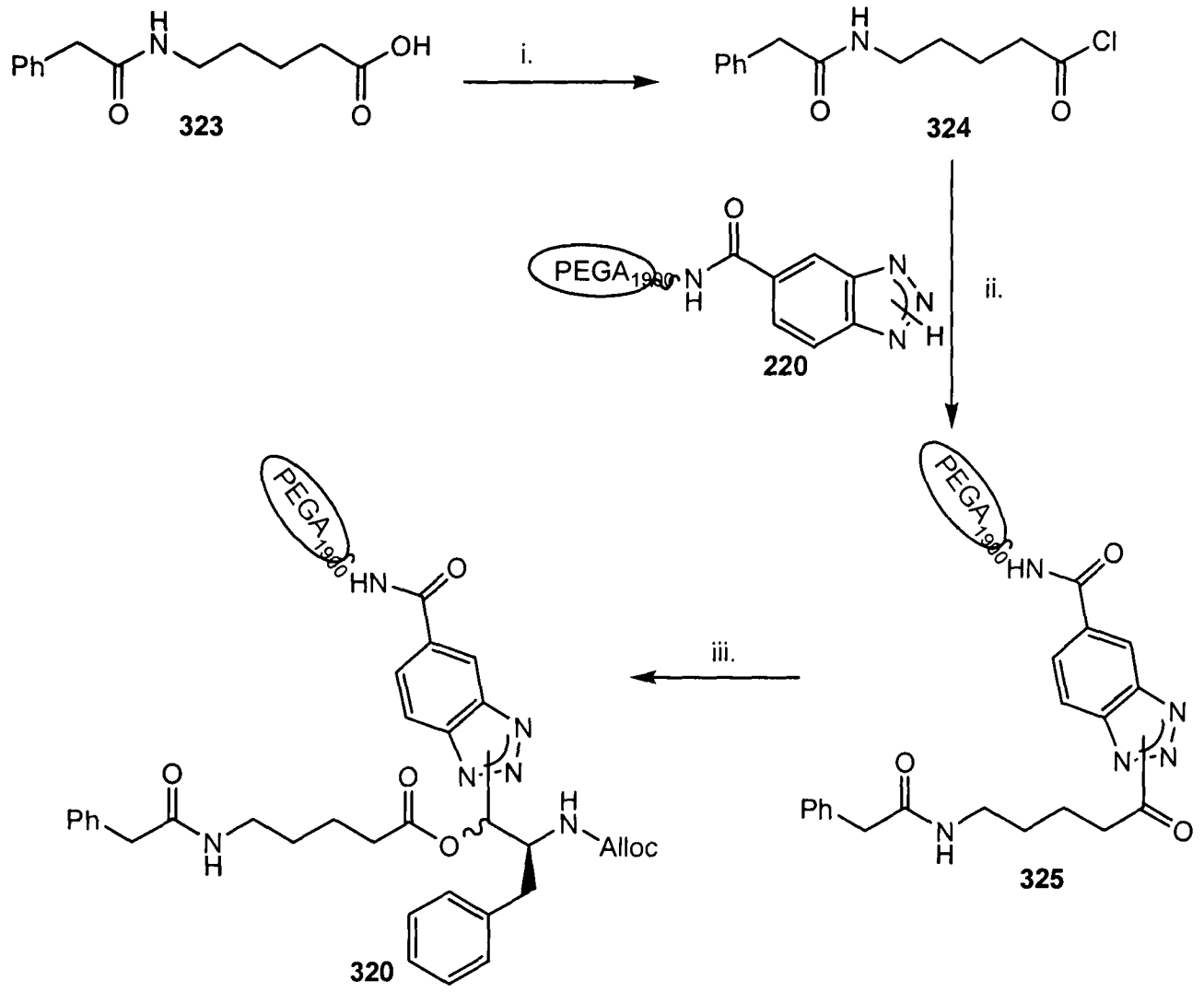
Scheme 93: *Reagents and conditions:* i. Penicillin G acylase

In view of the ease of synthesis of the Waldmann linker[44] (Scheme 93), it was thought that a modified version of this linker might provide a simple spacer for the acetal linker **320**. (Scheme 94). The proposed linker involves a phenylacetamide moiety, which on cleavage with penicillin acylase liberates a free amine functionality. Although this linker **320** did not possess the phenyl ring as mentioned above (Scheme 93), it was anticipated that the linker would cyclize to generate valerolactam **322** and the desired aldehyde **171**, according to Baldwins' rules.



Scheme 94: *Proposed safety catch linker **320** for enzymatic cleavage.*

The synthesis of the proposed safety catch linker **320** was envisaged using Katritzky methodology[110] as before (Scheme 95). Pentanoic acid **323** was prepared from 2-aminovaleric acid and phenylacetyl chloride in sodium hydroxide, in 50% yield. Treatment of pentanoic acid **323**, with oxalyl chloride and catalytic dimethylformamide, afforded the pentanoyl chloride **324** in moderate yield (30%). Coupling of the pentanoyl chloride **324** to $PEGA_{1900}$ benzotriazole resin **220** was carried out in base to give **325**. Subsequent aldehyde **171** insertion was achieved in the presence of base, to give the safety catch linker **320,** in 24% loading.



Scheme 95: *Reagents and conditions:* i. oxalyl chloride, cat. DMF, anhydrous DCM, 30%; ii. Et_3N, anhydrous DCM, 20 h, r.t.; iii. (*S*)-**171**, triethylamine, acetonitrile, 20 h, r.t., 24% loading.

Two small scale hydrolysis reactions were performed (~60 mg of **320**), along with two control reactions. Linker **320** was treated for 16 hours with the appropriate cleavage mixture (Table 6). The hydrolysis experiments were repeated twice more and the amount of phenylacetic acid/phenylacetamide released was quantified by reverse phase HPLC.

Cleavage method	**Cleavage Yield**
Control (10% acetonitrile/buffer pH 7.4)	-
Penicillin acylase in 10% acetonitrile/buffer pH 7.4	5%
(Control) Methanol	-
7M ammonia/methanol	100%

Table 6: *Results of hydrolysis experiments of **320**.*

From the results shown in Table 6, it can be seen that the safety catch linker **320** is a poor substrate for penicillin acylase (5%). The catalytic domain of penicillin acylase is located at the base of a large cavity,[128] therefore, the phenylacetyl moiety must be able to penetrate into this cavity. The pentyl spacer may aggregate due to

the high flexibility of the carbon chain and hence, render it incapable of penetrating into the deep cavity. It should be noted that, again the linker **320** was unstable in the phosphate buffer/10% acetonitrile control (10%; however, this was considered when calculating the enzyme hydrolysis yield. In all hydrolysis mixtures, allyloxycarbonyl-phenylalaninal **171** was detected in similar quantities to phenylacetic acid/phenylacetamide, which indicated that the safety catch linker fragmented as predicted (Scheme 94).

6.2.11 Summary and conclusions

Linkers **287-290** and **293** were prepared on solid support, by aldehyde insertion into the corresponding *N*-acyl benzotriazole moiety. Polystyrene bound 1-(benzotriazol-1-yl)alkyl hemiaminal **287** was prepared in 77% loading, which was much improved over the aminal linker analogue **232** (58%). An investigation into the chemical cleavage of the hemiaminal linkers **287** and **293** indicated that only sodium hydroxide/dioxane and methanolic ammonia were suitable for aldehyde cleavage; however, both methods completely epimerized the α-stereocentre of the cleaved aldehyde **173**. In addition, sodium hydroxide cleavage of **293** resulted in the formation of unwanted by-products.

The enzymatic hydrolysis of model $PEGA_{1900}$-bound hemiaminal linkers **288-290** were studied in order to gain an insight into what influence the solid support would have on the substrate specificities of the enzymes. In the case of The $PEGA_{1900}$ support was found to have a major detrimental effect on the substrate specificity of Hog pancreas lipase. In the case of **288**, 42% conversion yield was obtained for Hog pancreas lipase. In the solution phase analogue **273**, Hog pancreas lipase hydrolysed **273** in 95% yield. To conclude, it would seem that direct comparison between solution- and solid-phase analogues cannot be made in the case of enzymatic hydrolyses yields.

$PEGA_{1900}$-bound benzotriazole hemiaminals **295-297** were synthesized in a manner similar to the polystyrene benzotriazole linker **293**. Enzymatic hydrolysis

screening was carried out with a variety of enzymes. The best enzymatic cleavage yield was obtained with hemiaminal **296** and penicillin acylase (31%). This yield is comparable with the enzymatic cleavage of solution phase analogue, **279** (27%). This suggests that the substrate specificity of the enzyme plays a vital role in the enzymatic hydrolysis reactions. *Mucor javanicus* lipase hydrolysed linker **296** in 18% yield, which was much improved over the solution phase analogue cleavage **279** (1.4%). *Pseudomonas fluorescens,* Hog pancreas, *Chromobacterium viscosum* and *Penicillium roqueforti* lipase all hydrolysed **296** with minimal success (11-14%). Since, most of the enzymes screened showed some cleavage of **296,** this suggests that only a small amount of the support bound linker was accessible to the enzyme. Recleavage of the **296** with more enzyme did not result in any further hydrolysis. This implied that diffusion was not a limiting factor in the hydrolysis by enzymes. One possible use for the low enzymatic hydrolysis yields might be in biological screening. For example, in a two stage cleavage strategy, where 10-31% of the linker can be cleaved by biocatalysts and the remaining substrate on the linker can be cleaved using chemical methods for the identification of possible substrates for enzymes. A similar strategy has been exploited by Barany *et. al.*[24] who exploited the differences in properties of surface and interior sites of TentaGel resin to cleave only 10-15% of the substrate with enzyme, with the remaining sites available for identification of the active ligands.

Mucor javanicus lipase and penicillin acylase cleaved the linker **296** to afford (*S*)-allyloxycarbonyl-phenylalaninal **171** with enantiomeric excess of >98%.

The use of controlled pore glass (100 nm) as a support for the hemiaminal linker **301** resulted in no enzymatic hydrolysis with either *Mucor javanicus* lipase or penicillin acylase. This implied that the choice of resin was critical to the success of the enzymatic cleavage and that the resin should be treated as a co-solvent rather than as an inert carrier.

Product inhibition was dismissed as a possible problem in the hydrolysis of these linkers **295-297**. Allyloxycarbonyl-phenylalaninal **171** was screened for

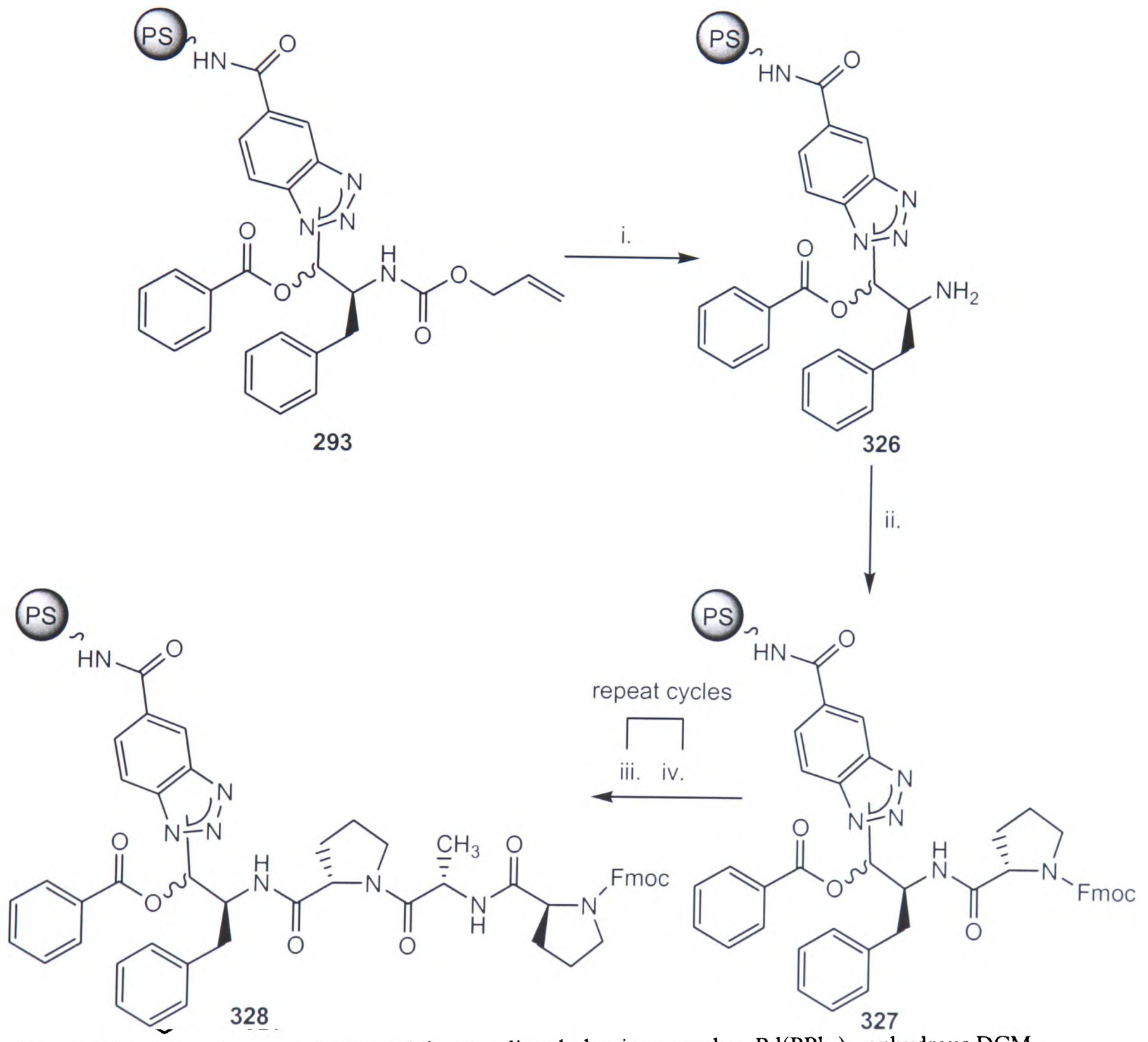
inhibition against the enzymes and it was found that **171** was not inhibiting any of the enzymes used.

The synthesis of a safety catch linker **320** was performed in order to minimize possible unwanted steric or electronic interactions between the linker/substrate and the biocatalyst; however, subsequent enzymatic hydrolysis with penicillin acylase did not improve the hydrolysis yields. This suggested that steric hinderence between the chiral centre of **295** and the solid support was not hindering the enzymatic hydrolysis.

To conclude, these results suggest that the hydrolysis of **296** was limited to some extent by the substrate specificity of the enzyme. It was also probable that, steric or electronic hinderance between the $PEGA_{1900}$ resin and enzyme is a major contributing factor in the low cleavage yields obtained. As a consequence of these unwanted interactions, the choice of solid support was crucial to the success of enzymatic hydrolysis; with $PEGA_{1900}$ offering the best enzymatic cleavage yields. Improved enzymatic hydrolysis yields might be obtained by tailoring of the resin to the enzyme. For example, by the use of charged PEGA supports. This concept was recently investigated by Ulijn *et. al.*,[129] who reported on the introduction of permanent positive or negative charges into the acrylamide backbone. They discovered that electrostatic repulsion between adjacent chains increased the swelling of the polymer, therefore increasing the pore size. In the case of $PEGA^{+}$ resin, a significant enhancement in penicillin acylase hydrolysis yields (<50% conversion) were obtained, compared to neutral PEGA (<15% conversion). This effect was explained by the increased protein accessibility due to better swelling and electrostatic attraction between the negatively charged penicillin acylase and the positive resin. Further work might include a study of these positively charged resins for the enzymatic hydrolysis of the hemiaminal linkers **295-297**.

7 Results and discussion - Solid-phase peptide aldehyde synthesis on hemiaminal linker

Allyloxycarbonyl protected resin **293** was treated with a combination of borane-dimethylamine complex and palladium catalyst. The deprotected resin **326** was coupled to (*S*)-Fmoc-proline using HBTU/HOBt in the presence of base. Two cycles of Fmoc deprotection and Fmoc-amino acid coupling resulted in the synthesis of a tetrapeptide aldehyde on solid support **328** (Scheme 96).



Scheme 96: *Reagents and conditions:* i. borane-dimethylamine complex, $Pd(PPh_3)_4$, anhydrous DCM, 10 min, r.t; ii. (*S*)-Fmoc-Pro-OH, DMF, DIPEA, 0.45 M HBTU/HOBt solution in DMF, 4 h, r.t.; iii. 20% piperidine in DMF, 20 min; iv. (*S*)-Fmoc-Ala-OH, DMF, DIPEA, 0.45 M HBTU/HOBt solution in DMF, 4 h, r.t.; v. 20% piperidine in DMF, 20 min; vi. Fmoc-AA-OH, DMF, DIPEA, 0.45 M HBTU/HOBt solution in DMF, 4 h, r.t.

The presence of tetrapeptide aldehyde **257** was confirmed by basic cleavage (1 M NaOH/dioxane) of **328**, followed by reverse phase HPLC and electrospray mass spectrometry analysis. In the HPLC trace (Figure 34), only two main products were observed; benzoic acid (7.3 min) and Fmoc-peptide aldehyde (9.0 min).

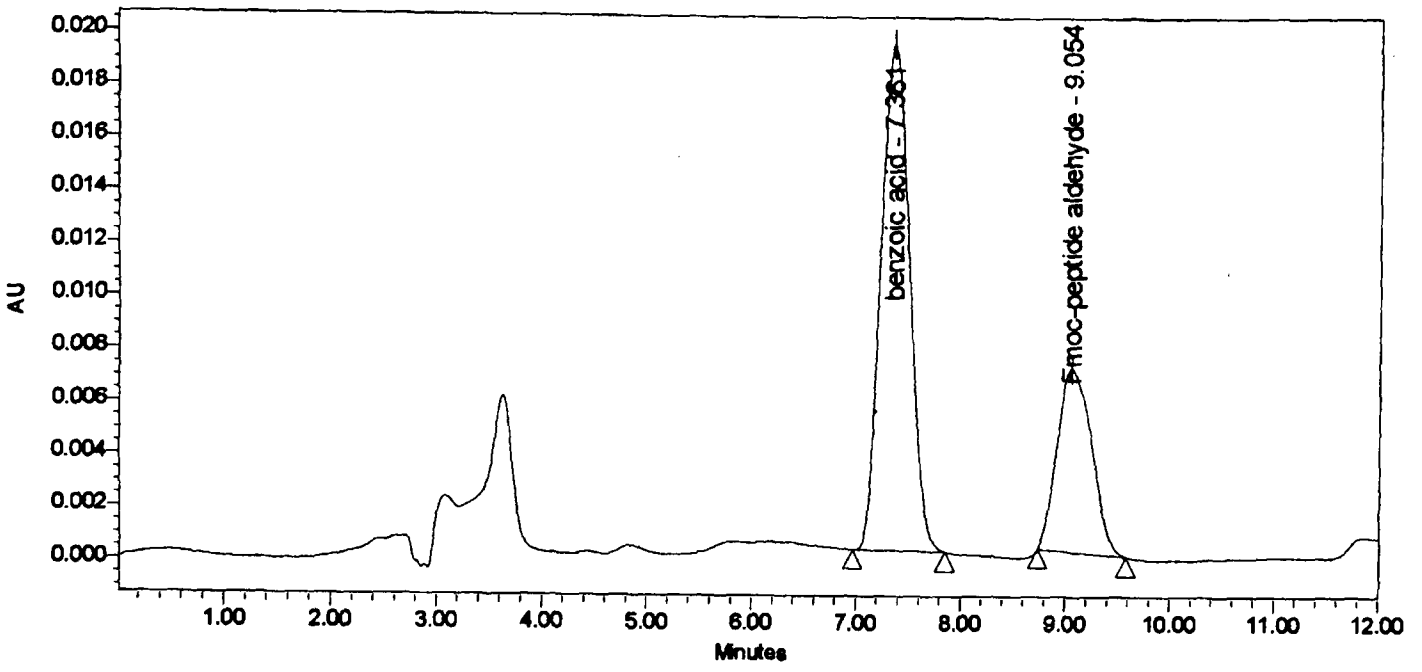


Figure 34: *Reverse phase HPLC trace of cleavage mixture of* ***328****.*

Semi-preparative HPLC was carried out to obtain a sample of peptide aldehyde **257** and the identity was confirmed by electrospray mass spectrometry (Figure 35). The desired molecular ion was observed at 637 *m/z*, along with MNa^+ and MK^+ ions at 659 and 675 *m/z* respectively. It was assumed that sodium hydroxide would completely epimerize the α-stereocentre of the peptide aldehyde **257**, therefore the ideal cleavage method would involve the use of enzymes.

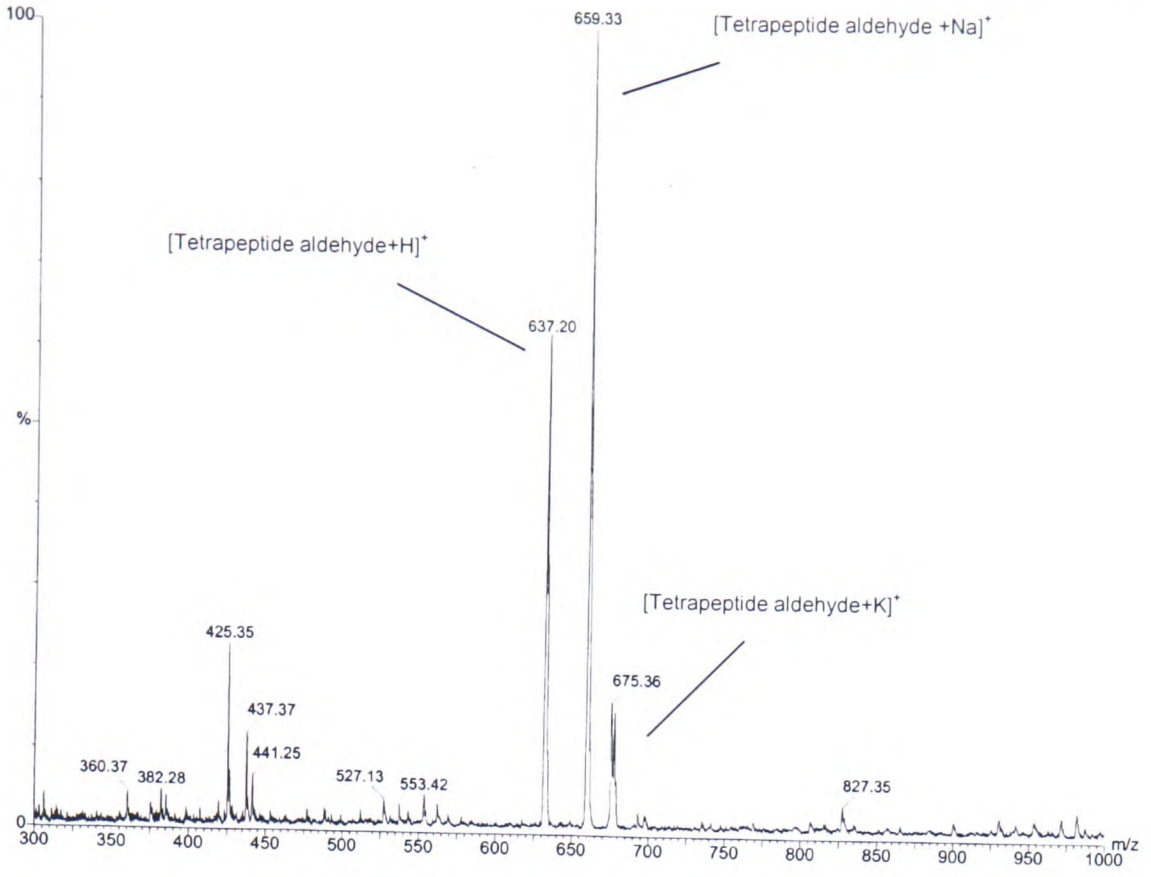


Figure 35: *Electrospray mass spectrometry of peak at 9.0 min in reverse phase HPLC trace (Figure 34).*

In order to study the use of hydrolytic enzymes for cleavage of these linkers, the solid support must be compatible with the specific conditions required for the biocatalyst. As mentioned previously, macroporous resins consist of a rigid open pore structure and access of liquid reagents to reaction sites occurs by rapid diffusion through the rigid, open pore structure rather than through a swollen gel phase.

Macroporous polystyrene-bound peptides **329** and **330**, were constructed in the same way as the polystyrene peptide **328** (Figure 36). The loadings achieved were 35% and 22% respectively.

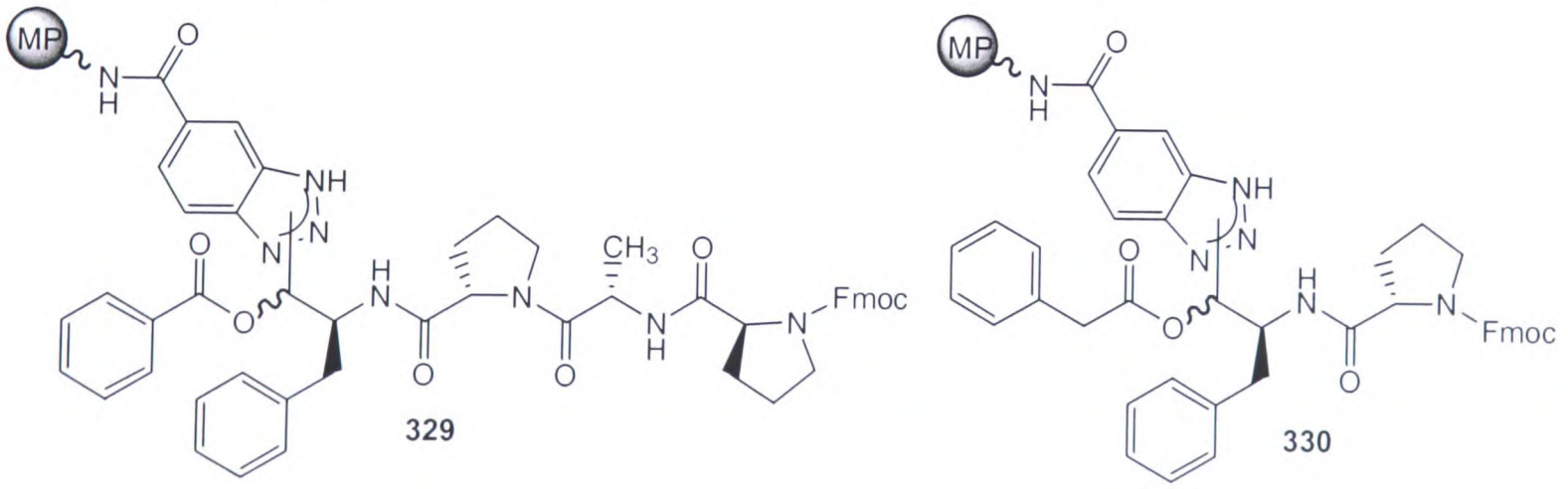


Figure 36: *Macroporous polystyrene supported peptide aldehydes **329** and **330**.*

Enzyme hydrolysis experiments were carried out by Rein Ulijn at Edinburgh University. Each substrate was treated with the appropriate enzyme (1 mg mL^{-1}) in phosphate buffer pH 7.4 for 16 hours and the amount of phenylacetic acid released was quantified by reverse phase HPLC (Section 8.2.9).

Enzyme	**Substrate**	
	329	**330**
Mucor Meihei lipase	5%	-
Penicillin acylase	1%	18%

Table 7: *Enzyme hydrolysis yields for **329** and **330**.*

From Table 7, it can be seen that the best cleavage was obtained with the phenylacetyl linker **330** and penicillin acylase. Benzoic linker **329** was not a substrate for penicillin acylase or *Mucor Meihei* lipase. It was concluded that macroporous polystyrene resin was not suitable as a support for the enzymes tested. It is probable that the hydrophobic polystyrene backbone repels the hydrophilic enzyme, thus limiting access to the active site of the enzyme.

7.1 Summary and conclusions

Tetrapeptide aldehyde **257** was synthesized on polystyrene linker **328** and cleaved from the solid support using sodium hydroxide. Purification of tetrapeptide aldehyde **257** by semi-preparative HPLC followed by electrospray mass spectrometry indicated the presence of tetrapeptide aldehyde **257**. In support of this evidence, tetra-peptide aldehyde **257** was also synthesized on polystyrene aminal linkers **250** and **256** and cleaved using acid to release **257**. Electrospray mass spectrometry of the cleavage mixtures gave the appropriate $[M+H]^+$ and $[M+Na]^+$ ions for the tetrapeptide aldehyde **257**.

Enzymatic hydrolyses of macroporous bound peptide aldehydes **329** and **330** were performed. Although, **329** was not hydrolysed by *Mucor meihei* lipase or penicillin acylase, **330** was cleaved by penicillin acylase in 18% yield. This relatively low hydrolysis yield was explained by the inability of hydrophilic enzyme to penetrate into the hydrophobic resin. Further work might include a study of

hydrolysis yields on positively charged $PEGA_{1900}$ bound analogues of these linkers **329** and **330**.

8 Experimental

8.1 General experimental

8.1.1 Instrumentation

^{1}H and ^{13}C NMR spectra were recorded on Bruker AC200, Bruker AC250, Varian Gemini 200 and Varian Inova 600 instruments. Chemical shifts (δ_H, δ_C) are reported in p.p.m. and coupling constants (*J*) are in hertz (Hz). *J* values are quoted to within one decimal place. *J* values for coupled protons are expected to match, however the small inconsistancies to be found in these results are due to the inaccuracy of the NMR machine and are within the expected error limits (±0.5). Magic angle spinning ^{1}H NMR was recorded on a Varian Inova 600 instrument.

Electrospray mass spectrometry (ES-MS) was performed using a Micromass platform II instrument with MassLynx software. Fast Atom Bombardment (FAB) mass spectrometry was performed using a Kratos MS50TC instrument.

Infrared spectroscopy (IR) was performed using a Perkin Elmer Paragon 1000 FT-IR spectrometer with the frequencies (ν) measured in wavenumbers (cm^{-1}). Solution samples were measured as thin films or neat. Solid samples were measured as nujol mulls or KBr discs. Solid resins were measured either swollen in DCM or as KBr discs.

Reaction optimization experiments were carried out on a Gilson SK233 system and analysed by reverse phase HPLC.

Optical rotations were measured on an Optical Activity AA-1000 polarimeter (Sodium 589 nm detection). Sample concentration was measured in g/100 mL and $[\alpha]^{20}_{D}$ values are quoted in 10^{-1} deg cm^{-1}.

Melting points were recorded on a Gallenkamp melting point apparatus and were uncorrected.

A 360° Stuart scientific SB1 blood rotator and a New Brunswick Scientific Gyrotory® water bath shaker were used for resin agitation at room temperature.

Elemental analysis was performed using a Perkin-Elmer 2400 CHN Elemental Analyser. Elemental analysis of resin bound compounds was carried out by Medac Ltd.

8.1.2 Chromatography

Analytical thin layer chromatography (TLC) was performed using Merck aluminium backed plates coated with silica gel $60F_{254}$. Identification was carried out using U.V. fluorescence (254 nm), *p*-anisaldehyde, ammonium molybdate, potassium permanganate and ninhydrin dips. Flash column chromatography was carried out with a variety of glass columns using BDH silica gel (40-63 μm) or a Biotage pre-packed cartridge system.

8.1.3 Reagents and solvents

All reagents were standard laboratory grade and used as supplied unless otherwise stated. Phenylacetamide was obtained and used as supplied from Maybridge plc. For HPLC methods: U.V. grade acetonitrile and water, HPLC grade *iso*-propanol, hexane and trifluoroacetic acid were used. Where a solvent has been described as anhydrous, it was either purchased as anhydrous grade or was distilled prior to use. Tetrahydrofuran was pre-dried over sodium wire and distilled from sodium benzophenone ketal. Peptide synthesis grade, dimethylformamide (DMF), was obtained from Rathburn. Methylene chloride-D_2 was acquired from Apollo Scientific Ltd.

8.1.4 Enzymes

All enzymes were purchased from Fluka or Sigma and the specific activities are shown below. Penicillin amidase was obtained from Sigma as a solution in 0.1 M phosphate buffer pH 7.5 (76 mg protein mL^{-1}, 23 units mg^{-1}).

Thermoanaerobium brockii esterase	1.7 U/g	*Penicillium roqueforti* lipase	151 U/ mg
Pseudomonas fluorescens lipase	33 U/ mg	*Rhizopus arrhizus* lipase	2 U/ g
Hog pancreas lipase	23.3 U/ mg	*Candida antarctica* lipase	3 U/ mg
Chromobacterium viscosum lipase	-	*Mucor meihei* lipase	1 U/ mg
Candida lipolytica lipase	1.1 U/ mg	*Candida cylindracea* lipase	2 U/ mg
Mucor javanicus lipase	5.2 U/ mg		

8.2 General experimental – solid phase

8.2.1 Resins

All resins were obtained from Novabiochem, Advanced Chemtech, Polymer Laboratories or CPG Inc (now Millipore Corporation).

8.2.2 Solid phase reactions

Solid phase reactions were carried out in 3, 10, or 25 mL plastic isolute SPE filtration columns with caps, luer tip caps and 20 μm porosity frits, purchased from Jones Chromatography and spun on a 360° blood rotator. Large scale reactions (>1 g resin) were performed in standard glass round bottom flasks and a New Brunswick Scientific Gyrotory® water bath shaker was used to agitate the resin. In general,

reactions were allowed to proceed for >15 h, before filtering and washing as described in Section 8.2.3

8.2.3 General washing protocol

All resins were washed according to the following procedure, unless otherwise stated: THF (x2), DMF (x2), DMF/MeOH (1:1) (x2), DMF (x2), THF (x2), DCM (x2). Resins were agitated during washing using plastic pipettes to minimize loss of polystyrene resin, which adheres to glass. Isolute tubes were filtered using a VacMaster 10 workstation, otherwise a standard sinter funnel and water pump were used. Resins were dried in a vacuum oven before being analysed as illustrated in Section 8.2.4. $PEGA_{1900}$ was stored swollen in MeOH or DCM, as drying was detrimental to the internal structure of the resin.

8.2.4 On-resin analysis of polymer-supported intermediates

8.2.4.1 IR spectroscopy

In general, samples were prepared by taking a pre-washed and dried aliquot of resin (~5-10 mg), swelling in DCM and pressing between a pair of NaCl plates. The broad C-Cl stretch from DCM masks any diagnostic peaks below ν_{max} ~820 cm^{-1}, but signals in the carbonyl region are generally very apparent. Some resins were prepared by taking a small aliquot (~5-10 mg), grinding with KBr (FT-IR grade) and pressing into a pellet.

8.2.4.2 Gel phase ^{13}C NMR spectroscopy

Samples were prepared by loading dry resin into an NMR tube and adding CD_2Cl_2 dropwise until the resin had swollen. Care must be taken to avoid any air bubbles or foreign matter which may give rise to distortions in the NMR spectrum. Due to the nature of gel phase spectra, quaternary carbons are often too weak or broad to be assigned correctly. Also, many polymers give rise to broad peaks which can usually be attributed to signals from the backbone (*e.g.* aromatic or benzylic), often obscuring any weaker signals in the vicinity. Therefore, where gel phase NMR

data has been quoted in the experimental section, peaks have been assigned as far as possible, often with the aid of relevant solution spectra. Where atoms are not assigned, it is assumed that the corresponding peaks have either been obscured or are too weak to assign confidently.

8.2.4.3 Magic angle spinning (MAS) ^1H NMR[121]

Samples were prepared by loading dry resin into a zirconium rotor (4mm diameter and slowly adding $CDCl_3$. This served two purposes. Firstly, the solvent caused the resin to swell, increasing the mobility of the attached molecule, giving sharper signals. Secondly, to allow locking of the deuterium signal to regulate the field position. When the sample is in place, it is tilted to the magic angle ($\theta = 54.7$) and is spun at rates up to 5 kHz. Due to the nature of some resins, some peaks are often very broad or are obscured by the polymer backbone. Therefore, peaks have been assigned as far as possible. Where atoms are not assigned, it is assumed that the corresponding peaks have either been obscured or are too weak to assign confidently. Unless otherwise stated, if magic angle spinning ^{1}H NMR has not been quoted, then the nanoprobe was not available at the time of production of the resin.

8.2.5 Qualitative detection of resin-bound free amines

8.2.5.1 Kaiser test[104]

A small sample of washed and dried resin (~2 mg) was treated with two drops of each reagent: A, B and C. The resin suspension was heated (~100 °C) for 2-5 min and any colour change noted. A dark blue/black colour indicated the presence of free amine, whereas a straw brown colour verified complete coupling. Due to the sensitivity of the test (5 μmole/ g free amine) if a pale blue colour developed after 5min it was assumed that the reaction had reached virtual completion, nevertheless, the test was used in conjunction with the TNBS test. (Section 8.2.5.2).

Reagents: A; ninhydrin (500 mg) in EtOH (10 mL)

B; phenol (80 g) in EtOH (20 mL)

C; 0.001 M potassium cyanide (2 mL) diluted to 98 mL with pyridine

8.2.5.2 TNBS test[130]

A small portion of washed and dried resin was treated with one drop of each solution: D and E. Red beads, viewed under a microscope, indicated the presence of free amine.

Reagents: D; 10% DIPEA in DMF

E; 1% 2,4,6-trinitrobenzenesulfonic acid in DMF.

8.2.6 Determination of loading

8.2.6.1 Fmoc analysis

A minimum of two samples of dry resin (4-10 mg) were weighed directly into 5 or 10 mL volumetric flasks and made up to the volumetric level with a solution of 20% piperidine in DMF. The samples were sonicated at room temperature for 2 min, before recording the U.V. spectrum of the supernatant between 280 and 320 nm. Where absorbances with $\lambda_{max} > 1$ were measured, the sample was diluted with blank 20% piperidine in DMF, until an absorbance of $\lambda_{max} < 1$ was achieved, in accordance with the Beer-Lambert Law. Equation 1 below was used to calculate the resin loading:

$$\textbf{mmol/ g} = (\textbf{A x v})/(\varepsilon \textbf{x} 10^{-3} \textbf{ x wt}) \qquad \textbf{(1)}$$

[**A** = absorbance at 301 nm (λ_{max}); **v** = volume (mL); **ε** = extinction coefficient of *N*-(9-fluorenylmethyl)piperidine[132] (ε (301 nm) = 7800 M^{-1} cm^{-1})]

Due to the errors incurred from weighing small samples and carrying out repeated dilutions, the loadings obtained are only considered to be accurate to ~±0.05 mmol g^{-1} and are merely intended to give an indication of the success of the reaction. In terms of starting loadings for reactions, although the loading of the resin to be derivatised was calculated where possible, in order to account for inconsistencies, reagents were often added with respect to the manufacturer loading.

8.2.6.2 Calculation of loading and yield of resin bound substrates

When determining the yield of a solid phase reaction, the weight gain/loss during the reaction must be accounted for, when calculating the maximum theoretical yield. The maximum theoretical loading (L_{max}) can be calculated using equation 2.

$$\mathbf{L_{max}\ (mmol\ g^{-1}) = L_{sm}/[1 + (L_{sm}\ x\ MWt)]\ \ x\ 1000} \qquad \mathbf{(2)}$$

[where $\mathbf{L_{sm}}$ = loading of the starting material (mol g^{-1}); **MWt** = molecular weight added to the resin (g)]

When the loading of a precursor is unknown, it is possible to calculate backwards to get the calculated loading of the starting material (L_{calc}) using equation 3.

$$\mathbf{L_{max}\ (mmol\ g^{-1}) = \ \ L_{prod}/[1 - (L_{prod}\ x\ MWt)]\ x\ 1000} \qquad \mathbf{(3)}$$

[where $\mathbf{L_{prod}}$ = loading of the product resin (mol g^{-1})]

The increase or decrease in weight of a resin upon reaction can also be used to ascertain the loading. The coupling efficiency (C.E.)/yield can be calculated as described in equation 4.

$$\mathbf{C.E.=[actual\ weight\ increase(g)/expected\ weight\ increase(g)]\ x\ 100} \qquad \mathbf{(4)}$$

[where, expected weight increase (g) = $\mathbf{L_{sm}}$ x **MWt** x initial weight of resin]

Elemental analysis can be used to determine the loading of a resin using equation 5. Elements such as carbon, hydrogen, nitrogen, sulfur, chlorine, bromine and iodine can be evaluated in this way.

$$\mathbf{Loading\ (mmol\ g^{-1}) = [(\%A/N_A)\ x\ 10]/MWt_A} \qquad \mathbf{(5)}$$

[where,%**A** = % of element (**A**) found by elemental analysis; $\mathbf{N_A}$ = expected number of elements (**A**) on resin; $\mathbf{MWt_A}$ = molecular weight of element **A**]

8.2.7 Product release and subsequent analysis

8.2.7.1 General procedure for acidic cleavage

An aliquot of dry resin was treated with a solution of TFA/DCM/H_2O (9:10:1) for 2 h. The resin was filtered, washed with DCM and the filtrates and washings combined and concentrated.

8.2.7.2 General procedure for basic cleavage

An aliquot of dry resin was treated with a solution of 7 N NH_3/MeOH or 1 M NaOH/dioxane (1:3) for 2 h. The resin was filtered, washed with CH_3CN/H_2O (1:1) (~10 mL) and the filtrates and washings combined and concentrated.

8.2.8 ES-MS/LC-MS

After cleavage (Sections 8.2.7.1/8.2.7.2), the crude product was dissolved in a solution of CH_3CN/H_2O. An aliquot was withdrawn and analysed by ES-MS using a direct infusion set-up, Micromass™ Platform II spectrometer with MassLynx version 2.3 software. When required, LC-MS analysis was carried out using a Waters™ 2690 separations module and a Waters™ 486 tunable absorbance detector measuring at 210nm. A Phenomenex Luna 3 μ C18(2) 50 x 1 cm column was used as the stationary phase eluting with CH_3CN/H_2O at a flow rate of 0.1 mL min^{-1}. Retention times (R_t) are quoted in minutes. Gradient elutions are shown below.

Time (min)	H_2O	CH_3CN
0	95%	5%
5	95%	5%
35	5%	95%
40	5%	95%
45	95%	5%
60	95%	5%

8.2.9 Reverse phase HPLC/normal phase chiral HPLC

After cleavage (Sections 8.2.7.1/8.2.7.2), the crude product was dissolved in a known amount of CH_3CN/H_2O or *iso*-propanol for normal phase HPLC. Analytical high performance liquid chromatography (HPLC) was performed using a Waters™ tunable 486 absorbance detector, Waters™ autosampler and a Waters™ 600 pump and controller or a Waters Alliance 2690 separations module with a Waters tunable 486 absorbance detector. These were managed by the Waters Millennium Chromatography software package. A Phenomenex Sphereclone 25 cm x 0.46 cm 5 μm ODS2 column was used for all reverse phase HPLC and either a Diacel Industries Chiracel OD 25 cm x 0.46 cm, OD-H 25 cm x 0.46 cm or Chiralpak AD 25 cm x 0.46 cm column were used for all normal phase chiral HPLC. Unless otherwise stated, all columns were operated at room temperature. In all cases, a flow rate of 1 mL min^{-1} was used and detection was at 210 nm for reverse and normal phase HPLC. Due to the number of methods developed the exact mobile phase composition is documented within the analytical data for the specific compound of interest. In the case of quantitative results being required, two standard solutions were run for each compound to construct a standard concordance test. Retention times (R_t) are quoted in minutes.

8.3 Synthesis of amino aldehydes

8.3.1 General procedure for the preparation of Alloc-protected amino acids

Two methods were employed for the allyloxycarbonyl-protection of amino acids.

8.3.1.1 Allyl chloroformate method[131]

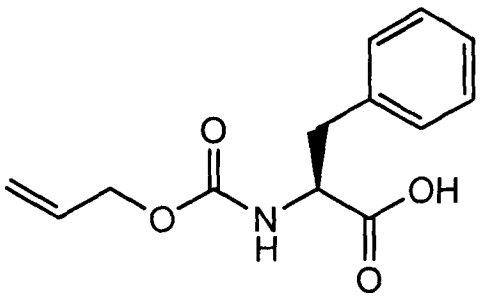
To a solution of the appropriate amino acid (1eq.) in 4 M sodium hydroxide was added in eight portions, allyl chloroformate (1eq.) in 4 M sodium hydroxide with stirring at 0 °C. The reaction was kept alkaline throughout. After the last addition, the reaction mixture was stirred for 15 min, and then allowed to stand at room

temperature for 15 min. After this time, the mixture was extracted with diethyl ether and the aqueous layer acidified with concentrated hydrochloric acid. A solid precipitated after standing at room temperature for 40 min. The solid was collected and dried over phosphorous pentoxide *in vacuo* for overnight.

8.3.1.2 Diallyl pyrocarbonate method[132]

To a solution of the appropriate amino acid (1eq.) in H_2O/Dioxane (3:2) at reflux, was slowly added diallyl pyrocarbonate (1eq.). After 30 min, the mixture was extracted with diethyl ether. The ethearal extracts were washed with brine, dried over magnesium sulfate and concentrated to give the product.

8.3.2 2-(S)-Allyloxycarbonylamino-3-phenyl-propionic acid 167[131,132,140]

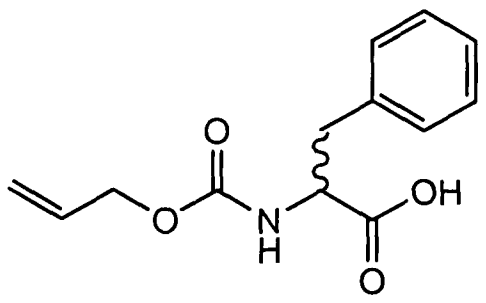


The general procedure 8.3.1.1 was followed with (*S*)-(-)-phenylalanine **166** (50.1 g, 303 mmol) in 4 M NaOH (77.5 mL), allyl chloroformate (32 mL, 0.30 mol) in 4 M NaOH (77.5 mL) to provide the title compound as a colourless solid (64.9 g, 86%). Alternatively, general procedure 8.3.1.2 was followed with (*S*)-(-)-phenylalanine **166** (0.44 g, 2.7 mmol), diallyl pyrocarbonate (0.50 g, 0.4 mmol) in water/dioxane (3:2) (6 mL) to give the desired product as a colourless oil (0.41 g, 61%). Both methods afforded product of sufficient purity to be used without any further purification. Analysis was comparable for both methods.

Mpt 91-96 °C, Lit[131] Mp 83-84 °C; R_f 0.73 [methanol/chloroform (2:1)]; υ_{max} (nujol)/ cm^{-1} 3320 (OH), 3061 (CH, aryl), 2725, 2670 (OH, carboxylic acid), 1695 (CO, carboxylic acid and urethane), 1603 (C=C, arom), 1541 (CO, amide II), 1495 (C=C, arom); δ_H ($CDCl_3$; 250 MHz) 3.15 (1H, dd, *J*=13.9, 6.3 Hz, C*H*$_a$CH$_b$Ph), 3.26 (1H, dd, *J*=13.8, 5.3, CH$_a$C*H*$_b$Ph), 4.61 (2H, d, *J*=5.5 Hz, C*H*$_2$O), 4.75 (1H, m, C*H*CH-$_a$CH$_b$), 5.26 (1H, dd, *J*=11.2, 1.3 Hz, C*H*$_{cis}$CH$_{trans}$), 5.32 (1H, dd, *J*=18.6, 1.4 Hz, CH$_{cis}$C*H*$_{trans}$), 5.91 (1H, ddt, *J*=19.0, 11.0, 5.7 Hz, C*H*=CH$_2$), 6.25 (1H, d, *J*=7.8 Hz, N*H*), 7.13-7.25 (5H, m, Ph); δ_C ($CDCl_3$; 63 MHz) 37.6 (*C*H$_2$Ph), 55.6 (*C*HCH$_2$), 65.7

(CH_2O), 117.6 (CH_2=CH), 126.7 (CH_{arom}), 128.4 (CH_{arom} x 2), 129.2 (CH_{arom} x 2), 132.4 (CH=CH_2), 136.4 (C_{arom}), 156.2 (C=$O_{urethane}$), 176.8 (C=O_{acid}); $[\alpha]_D^{22}$+34.5° (0.01, $CHCl_3$), Lit $[\alpha]_D^{22}$+35.8° (0.01, $CHCl_3$); FAB-MS, Found: MH^+ 250.1071, $C_{13}H_{16}NO_4$ requires MH^+ 250.1072.

8.3.3 2-(R/S)-Allyloxycarbonylamino-3-phenyl-propionic acid 169[131,132,140]

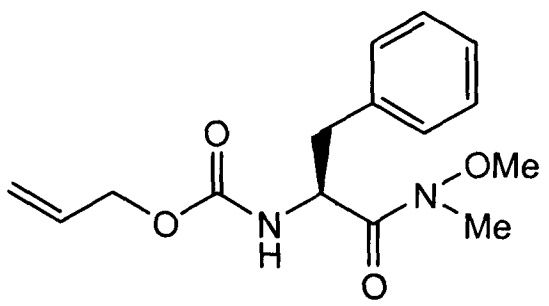


The general procedure 8.3.1.1 was followed with (*R/S*)-phenylalanine **168** (3.0 g, 18 mmol) in 4 M NaOH (5 mL), allyl chloroformate (1.9 mL, 18 mmol) in 4 M NaOH (5 mL) to provide the desired compound as a colourless solid (3.6 g, 80%). The product was obtained in sufficient purity to be used without further purification. Analysis comparable to 8.3.2 except Mp 77-80 °C Lit[131] Mp 83-84 °C

8.3.4 General procedure for the preparation of Weinreb amides

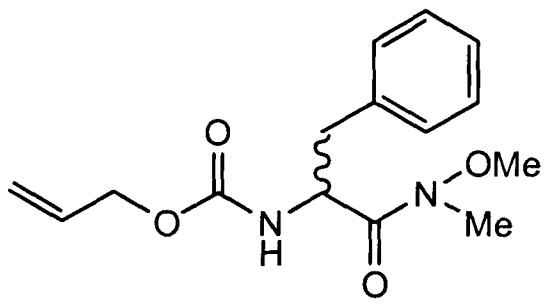
Diisopropylethylamine (1.2eq.) was added to a solution of Alloc-protected phenylalanine (1eq.) in dimethylformamide. HBTU (1.1eq.) and HOBt (1.1eq.) was added and after a few minutes, *N,O*-dimethyl-hydroxylamine.hydrochloride (1.5eq.) was added with diisopropylethylamine (1.4eq.). After 90 min, water (40 mL) was added and the reaction mixture was extracted with diethyl ether (3 x 50 mL). The organic extracts were combined and washed with water (2 x 50 mL), 2M HCl (3 x 50 mL), saturated $NaHCO_3$ (3 x 50 mL) and brine (2 x 50 mL). The organic layer was dried over magnesium sulfate and concentrated to give the title compound.

8.3.5 Allyl (S)-1-(N-methoxy-N-methylcarbamoyl)-2-phenylethylcarbamate 170



The general procedure 8.3.4 was followed with diisopropylethylamine (0.89 mL, 5.1 mmol, 1.2eq.), (*S*)-Alloc-protected phenylalanine **167** (1.1 g, 4.3 mmol, 1eq.) in dimethylformamide (20 mL), HBTU (1.9 g, 4.7 mmol, 1.1eq.) and HOBt (0.63 g, 4.7 mmol, 1.1eq.); then *N,O*-dimethyl-hydroxylamine.hydrochloride (0.62 g, 6.4 mmol, 1.5eq.) and diisopropylethylamine (1.1 mL, 5.9 mmol, 1.4eq.). The product was obtained as a pale yellow oil (0.93 g, 75%). The compound was found to be enantiomerically pure (>99% e.e) by chiral HPLC, eluting using an isocratic Hexane/IPA/TFA (90:10:0.01) over 31 min at 5 °C; (*S*)-enantiomer at R_t = 19.3 min. R_f 0.67 [ethyl acetate/hexane (2:1)]; υ_{max} (film)/ cm^{-1}, 3086, 3062 (CH, arom), 3028, 2978, 2940 (CH, sat), 1720 (CO, urethane), 1651 (CO, amide I), 1604 (C=C, arom), 1531 (CO, urethane, amide II); δ_H ($CDCl_3$; 250 MHz) 2.89 (1H, dd, *J*=14.0, 7.4 Hz, C*H*$_a$CH$_b$Ph), 3.05 (1H, dd, *J*=14.0, 6.1, CH$_a$C*H*$_b$Ph), 3.16 (3H, s, NC*H*$_3$), 3.66 (3H, s, OC*H*$_3$), 4.50 (2H, dd, *J*=5.5, 1.4 Hz, OC*H*$_2$), 4.96-5.01 (1H, m, C*H*CH$_a$CH$_b$), 5.16 (1H, dd, *J*=10.4, 1.3 Hz, C*H*$_{cis}$CH$_{trans}$), 5.24 (1H, dd, *J*=17.0, 1.4 Hz, CH$_{cis}$C*H*$_{trans}$), 5.39 (1H, d, *J*=8.5 Hz, NH), 5.86 (1H, ddt, *J*=17.0, 10.4, 5.3 Hz, C*H*CH$_2$), 7.13-7.31 (5H, m, Ph); δ_C ($CDCl_3$; 63 MHz) 31.9 (NC*H*$_3$), 38.5 (*C*H$_2$Ph), 51.9 (*C*HCH$_2$), 61.4 (OC*H*$_3$), 65.5 (OC*H*$_2$), 117.5 (*C*H$_2$=CH), 126.8 (*C*H$_{arom}$), 128.3 (*C*H$_{arom}$ x 2), 129.3 (*C*H$_{arom}$ x 2), 132.5 (*C*H=CH$_2$), 136.2 (*C*$_{arom}$), 155.5 (*C*=O$_{urethane}$), 171.8 (*C*=O$_{amide}$); $[\alpha]_D^{22}$ +27° (c. 0.01, $CHCl_3$); FAB-MS, Found: MH^+ 293.1500, $C_{15}H_{21}N_2O_4$ requires MH^+ 293.1501.

8.3.6 *Allyl (R/S)-1-(N-methoxy-N-methylcarbamoyl)-2-phenylethylcarbamate 172*



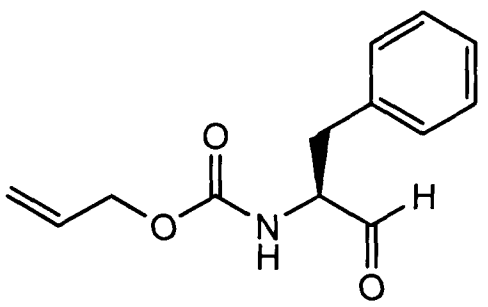
The general procedure 8.3.4 was followed with diisopropylethylamine (2.77 mL, 15.9 mmol, 1.2eq.), (*R/S*)-Alloc-protected phenylalanine **169** (3.30 g, 13.2 mmol, 1eq.) in dimethylformamide (66 mL), HBTU (5.79 g, 14.8 mmol, 1.1eq.) and HOBt (1.97 g, 14.8 mmol, 1.1eq.); then *N,O*-dimethyl-hydroxylamine.hydrochloride (1.94 g, 19.8 mmol, 1.5eq.) and diisopropylethylamine (3.23 mL, 18.4 mmol, 1.4eq.). The product was obtained as a colourless solid (3.3 g, 86%). The racemic compound (1:1 mixture of enantiomers) was analysed by chiral HPLC eluting using an isocratic Hexane/IPA/TFA (90:10:0.01) over 31 min at 5 °C; (*R*)-enantiomer at R_t = 14.7 min, (*S*)-enantiomer at R_t = 19.3 min.

Analysis comparable to 8.3.5

8.3.7 *General procedure for the preparation of Alloc-protected amino aldehydes*

To a solution of lithium aluminium hydride (3eq.) in tetrahydrofuran was added drop-wise a solution of Weinreb amide (1eq.) in tetrahydrofuran at -20 °C over 5 min. The reaction was stirred at -20 °C for 45 min. After this time, saturated $KHSO_4$ was added dropwise until effervescence ceased and a grey solid was produced. The mixture was stirred at room temperature for 50 min before removing the grey solid by filtration. The mother liquors were washed with saturated $KHSO_4$ and brine and dried over magnesium sulfate, then concentrated to yield the desired product.

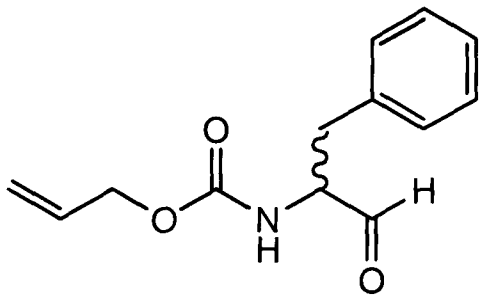
8.3.8 *Allyl (S)-1-formyl-2-phenylethylcarbamate 171*[138]



General procedure 8.3.7 was followed with lithium aluminium hydride (0.65 g, 17 mmol) in tetrahydrofuran (22 mL) and Weinreb amide **170** (1.65 g, 5.65 mmol) in anhydrous tetrahydrofuran (11 mL). The title compound was obtained as a pale yellow solid (1.0 g, 78%). The aldehyde was reduced to the corresponding alcohol using procedure 8.3.18 and was found to be enantiomerically pure (98% e.e.) by chiral HPLC, eluting using an isocratic Hexane/IPA (98:2) over 50 min at 10 °C; (*S*)-enantiomer at R_t = 30.5 min.

R_f 0.75 [ethyl acetate/hexane (2:1)]; υ_{max} (film)/ cm^{-1}, 3086, 3062 (CH, arom), 3028, 2940 (CH, sat), 1694 (CO, aldehyde), 1647 (CO, urethane), 1604 (C=C, arom), 1528 (CO, amide II); δ_H ($CDCl_3$; 250 MHz) 3.13 (2H, d, *J*=6.1 Hz, CH_2Ph), 4.50-4.53 (1H, m, CHCH$_2$), 4.56 (2H, dd, *J*=5.6, 1.4 Hz, OCH_2), 5.21 (1H, dd, *J*=10.4, 1.4 Hz, CH_{cis}CH$_{trans}$), 5.28 (1H, dd, *J*=18.6, 1.4 Hz, CH$_{cis}$CH_{trans}), 5.89 (1H, ddt, *J*=18.5, 10.6, 5.3 Hz, CHCH$_2$), 7.11-7.34 (5H, m, Ph), 9.50 (1H, s, CHO); δ_C ($CDCl_3$; 63 MHz) 35.3 (*C*H$_2$Ph), 60.9 (*C*HCH$_2$), 65.8 (O*C*H$_2$), 117.9 (*C*H$_2$=CH), 127.1(*C*H$_{arom}$), 128.7 (*C*H$_{arom}$ x 2), 129.2 (*C*H$_{arom}$ x 2), 132.3 (*C*H=CH$_2$), 135.2 (*C*$_{arom}$), 155.6 (*C*=O$_{urethane}$), 198.8 (*C*=O$_{aldehyde}$); $[\alpha]_D^{22}$ +80.0° (0.01, $CHCl_3$); FAB-MS, Found: MH^+ 234.1130, $C_{13}H_{16}NO_3$ requires MH^+ 234.1130.

8.3.9 *Allyl (S/R)-1-formyl-2-phenylethylcarbamate 173*[138]



General procedure 8.3.7 was followed using lithium aluminium hydride (0.40 g, 11 mmol) in tetrahydrofuran (13.3 mL) and Weinreb amide **172** (1.0 g, 3.6 mmol) in

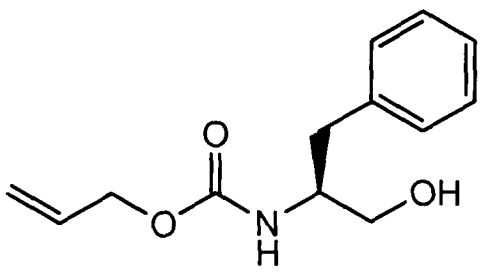
tetrahydrofuran (6.7 mL). The title compound was obtained as a pale yellow solid (0.73 g, 87%). The aldehyde was reduced to the corresponding alcohol using procedure 8.3.18 and analysed by chiral HPLC (1:1 mixture of enantiomers), eluting using an isocratic Hexane/IPA (98:2) over 50 min at 10 °C; (*S*)-enantiomer at R_t = 30.5 min, (*R*)-enantiomer at R_t = 35.5 min.

Analysis comparable to 8.3.8.

8.3.10 General Procedure for the preparation of amino alcohols[96]

To a cold (-15 °C) solution of Alloc-protected amino acid (1eq.) in 1,2-dimethoxyethane (DME), were successively added *N*-methyl morpholine (1eq.) and isobutyl chloroformate (1eq.). After 5 min, the precipitated *N*-methyl morpholine hydrochloride was removed by filtration, washed with DME (3 x 5 mL) and the filtrate and washings were combined in a large flask in an ice-salt bath. A solution of sodium borohydride (1.5eq.) in water was added at once, producing a strong evolution of gas, followed by water. The mixture was extracted with ethyl acetate; then the combined organic extracts with washed with water and brine, then dried over magnesium sulfate to afford the title compound.

8.3.11 Allyl (S)-1-hydroxy-3-phenylpropan-2-ylcarbamate 174[138]

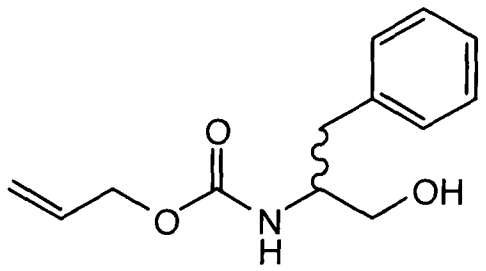


The general procedure 8.3.10 was followed with (*S*)-Alloc-protected phenylalanine **167** (2.03 g, 8.14 mmol) in DME (6.8 mL); and *N*-methyl morpholine (0.89 mL, 8.1 mmol) and isobutyl chloroformate (1.05 mL, 8.14 mmol). Then, sodium borohydride (0.46 g, 12 mmol) and water (3.3 and 168 mL) was added. The crude product was purified on a Biotage 40M cartridge eluting with ethyl acetate/hexane (2:1) to afford the pure compound as a colourless solid (0.63 g, 33%).

Chiral HPLC using an isocratic hexane/IPA (98:2) at 10 °C, confirmed the enantiomeric purity (98% e.e.) of the pure product; (*S*)-enantiomer at R_t = 33 min.

R_f 0.55 [ethyl acetate/hexane (2:1)]; Mp 54-55 °C; υ_{max} (film)/ cm^{-1} 3300 (OH), 3082, (CH, arom), 3029, 2951, 2856 (CH, sat), 1687 (CO, urethane), 1602 (C=C, arom), 1546 (CO, amide II); δ_H ($CDCl_3$; 250 MHz) 2.75 (2H, d, *J*=7.2 Hz, C*H*$_2$Ph), 3.45 (1H, dd, *J*=10.9, 5.0 Hz, C*H*$_a$CH$_b$OH), 3.55 (1H, dd, *J*=10.9, 3.6 Hz, CH$_a$C*H*$_b$OH, 3.81 (1H, m, C*H*CH$_2$Ph), 4.42 (2H, dd, *J*=5.6, 1.4 Hz, OC*H*$_2$), 4.92 (1H, d, *J*=7.9 Hz, NH), 5.07 (1H, dd, *J*=10.4, 1.3 Hz, C*H*$_{cis}$CH$_{trans}$), 5.15 (1H, dd, *J*=17.3, 1.4 Hz, CH$_{cis}$C*H*$_{trans}$), 5.75 (1H, ddt, *J*=17.2, 10.4, 5.5 Hz, C*H*CH$_2$), 7.08-7.20 (5H, m, Ph); δ_C ($CDCl_3$; 63 MHz) 37.8 (*C*H$_2$Ph), 54.5 (*C*HCH$_2$Ph), 64.4 (O*C*H$_2$), 66.1 (*C*H$_2$OH), 118.2 (*C*H$_2$=CH), 127.1(*C*H$_{arom}$), 129.1 (*C*H$_{arom}$ x 2), 129.7 (*C*H$_{arom}$ x 2), 133.1 (*C*H=CH$_2$), 137.9 (*C*$_{arom}$), 156.8 (*C*=O$_{urethane}$); $[\alpha]_D^{22}$ 36.0° (0.01, MeOH); *m/z* (ES-MS; +ve), 236 (50%, MH^+), 258 (100%, MNa^+); FAB-MS, Found: MH^+ 236.1282, $C_{13}H_{18}NO_3$ requires MH^+ 236.1286. Found C, 66.14%; H, 7.08%; N, 5.83. Requires C, 66.35%; H, 7.29%; N, 5.95%.

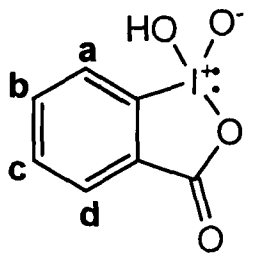
8.3.12 Allyl (R/S)-1-hydroxy-3-phenylpropan-2-ylcarbamate 175[138]



The general procedure 8.3.10 was followed with (*R/S*)-Alloc-protected phenylalanine **169** (1.00 g, 4.04 mmol) in 1,2-dimethoxyethane (DME) (6.8 mL); and *N*-methyl morpholine (0.44 mL, 4.0 mmol) and isobutyl chloroformate (0.52 mL, 4.0 mmol). Then, sodium borohydride (0.23 g, 6.1 mmol) and water (1.6 and 84 mL). The crude compound was obtained as a colourless solid (0.80 g, 84%) and used without further purification. Chiral HPLC using an isocratic hexane/IPA (98:2) at 10 °C, confirmed that the product was racemic; (*S*)-enantiomer at R_t = 33 min, (*R*)-enantiomer R_t = 36 min.

Analysis comparable to 8.3.11.

8.3.13 2-Iodoxybenzoic acid (IBX) 177[97]

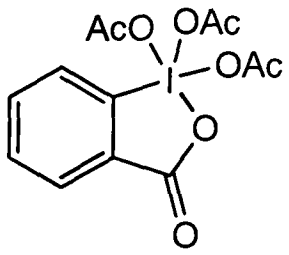


CAUTION! IBX is explosive under impact or heating to > 200 °C

2-Iodobenzoic acid **176** (25 g, 0.11 mol, 1eq.) was added all at once to a solution of oxone [$2KHSO_5$-$KHSO_4$-K_2SO_4] (185 g, 0.300 mol, 3eq.) in de-ionised water (1000 mL). The suspension was set at 70 °C; after 1h at this temperature a clear solution was obtained that delivered IBX upon cooling the solution to 0 °C for 2h and filtering and washing the crystals with water (6 x 50 mL) and acetone (2 x 50 mL). The colourless solid was dried *in vacuo* (29 g, 96%).

R_f baseline [ethyl acetate/hexane (8:2)]; Mp 228-230 °C decomposed, Lit[97] Mp 233 °C dec; υ_{max} (film)/ cm^{-1}, 1630 (CO); δ_H (DMSO-d_6; 250 MHz) 8.10 (1H, t, J=7.0 Hz, CH_b), 8.26 (1H, td, J=7.0, 1.0 Hz, CH_c), 8.26 (1H, d, J=7.3 Hz, CH_a), 8.40 (1H, d, CH_d); Found C, 29.61%; H, 1.59%; N, 0%. Requires C, 30.03%; H, 1.80%; N, 0%.

8.3.14 Dess-Martin periodinane 158[98]



177 (1.8 g, 6.5 mmol, 1eq.) was added to a solution of acetic anhydride (7.27 mL, 77.4 mmol, 12eq.) and $TsOH.H_2O$ (0.091 g, 0.48 mmol, 5 wt%). The flask was equipped with a drying tube and was heated at 80 °C for 2 h. After this time, the reaction mixture was cooled in an ice-water bath. The cold mixture was filtered and washed with anhydrous ether. The resulting colourless solid was quickly transferred to an argon filled flask and stored in the freezer (2.2 g, 80%).

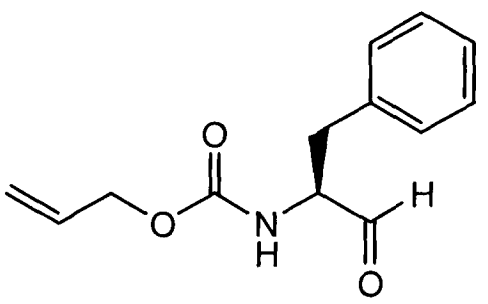
Mp 128-131 °C, Lit[98] Mp 134 °C; δ_H (DMSO-d_6; 250 MHz) 2.08 (9H, s, CH_3), 8.01 (1H, t, J=7.3 Hz, CH_{arom}), 8.11-8.33 (3H, m, CH_{arom}); δ_C (DMSO-d_6; 63 MHz) 21.9

(CH_3), 125.4 (CH_{arom}), 130.9 (CH_{arom}), 133.8 (CH_{arom}), 134.3(CH_{arom}), 147.4 (C_{arom} x 2), 168.4 ($C{=}O_{lactone}$), 172.9 ($C{=}O_{ester}$).

8.3.15 General procedure for preparation of amino aldehydes from alcohol[99]

Dess-Martin periodinane **158** (2.1eq.) was added to a solution of Alloc-protected phenylalaninol (1eq.) in water-saturated dichloromethane. The resulting suspension was stirred at room temperature and monitored by TLC. When no remaining alcohol was detected (~40 min), the reaction mixture was diluted with diethyl ether and a solution of sodium thiosulfate (11eq.) in saturated aqueous sodium bicarbonate was added. The mixture was stirred rapidly for 10 min, until both phases were clear. The layers were separated and the aqueous layer extracted with diethyl ether. The combined organic layers were washed with saturated aqueous sodium bicarbonate, water and brine, then dried over sodium sulfate. Concentration afforded the crude product.

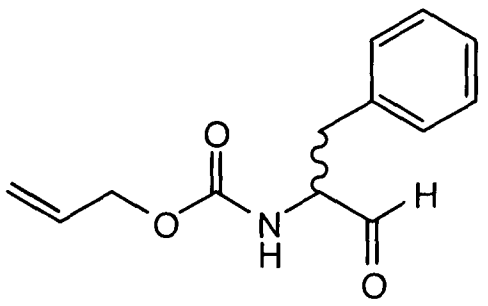
8.3.16 Allyl (S)-1-formyl-2-phenylethylcarbamate 171[138,139]



General procedure 8.3.15 was followed with **158** (9.32 g , 21.9 mmol) and **174** (2.46 g, 10.5 mmol) in water-saturated dichloromethane (9.5 mL); then a solution of sodium thiosulfate (17.6 g, 111 mmol) in saturated aqueous sodium bicarbonate (58 mL) was added. Concentration afforded the crude product as a colourless solid (1.9 g, 79%). The aldehyde was reduced to the corresponding alcohol using procedure 8.3.18 and enantiomerically pure (>99% e.e.) alcohol was analysed by chiral HPLC, eluting using an isocratic Hexane/IPA (98:2) over 50 min at 10 °C; (*S*)-enantiomer at R_t = 35.4 min.

Analysis comparable to 8.3.8 except R_f 0.60 [ethyl acetate/hexane (1:1)]; *m/z* (ES-MS; +ve), 234 (100%, MH^+); $[\alpha]_D^{22}$ +84.0° (c. 0.01, $CHCl_3$).

8.3.17 *Allyl (S/R)-1-formyl-2-phenylethylcarbamate 173*[138,139]



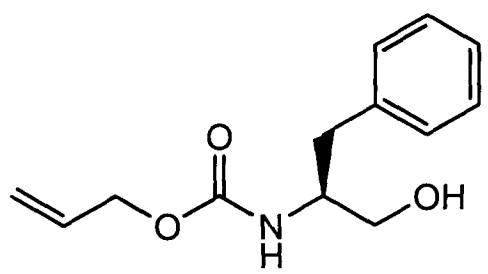
General procedure 8.3.15 was followed with **158** (1.62 g, 3.82 mmol) and **175** (0.43 g, 1.8 mmol) in water-saturated dichloromethane (3 mL); then a solution of sodium thiosulfate (3.6 g, 23 mmol) in saturated aqueous sodium bicarbonate (12 mL) was added. Concentration afforded the crude product as a colourless solid (0.26 g, 61%).

Analysis comparable to 8.3.8.

8.3.18 *General procedure for reduction of amino aldehydes for analysis*[133]

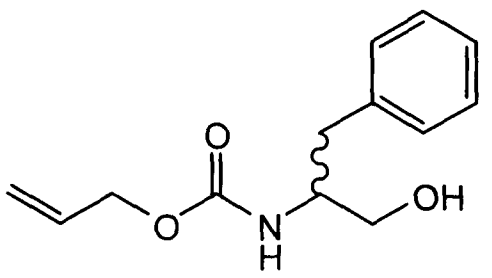
Alloc protected phenylalaninal (1eq.) was dissolved in ethyl acetate. Sodium borohydride (2eq.) was added and the solution stirred at room temperature for 30 min. After this time, the reaction mixture was poured into water, the organic layer was separated and the aqueous layer was extracted with ethyl acetate. The combined organic layers were dried over magnesium sulfate and concentrated to yield the crude product which was used without further purification.

8.3.19 *Allyl (S)-1-hydroxy-3-phenylpropan-2-ylcarbamate 174*[138]



General procedure 8.3.18 was followed with (*S*)-Alloc-phenylalaninal **171**. Chiral HPLC using an isocratic hexane/IPA (98:2) at 10 °C, confirmed the enantiomeric purity (98% e.e.) of the pure product; (*S*)-enantiomer at R_t = 33 min.

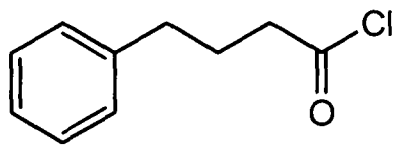
8.3.20 Allyl (R/S)-1-hydroxy-3-phenylpropan-2-ylcarbamate 175[138]



General procedure 8.3.18 was followed with (*R*/*S*)-Alloc-phenylalaninal **173**. Chiral HPLC using an isocratic hexane/IPA (98:2) at 10 °C, confirmed that the product was racemic; (*S*)-enantiomer at R_t = 33 min, (*R*)-enantiomer R_t = 36 min.

8.4 Solution phase synthesis of linkers

8.4.1 4-Phenyl-butyryl chloride[137]



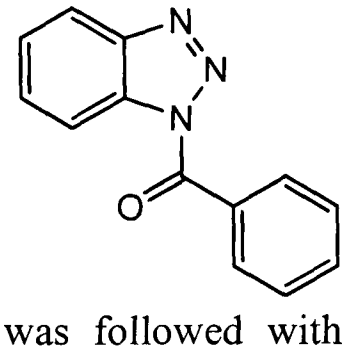
To a solution of 4-phenylbutyric acid (10.0 g, 61.0 mmol) in anhydrous dichloromethane (100 mL) was added oxalyl chloride (6.37 mL, 9.27 g, 73.0 mmol), followed by catalytic dimethylformamide (1 drop). The reaction was allowed to stir at room temperature for 1 h, evaporated to remove any excess oxalyl chloride, to provide the crude product as a moisture sensitive colourless oil. The crude product was purified by distillation at 120 °C/0.2 mm Hg (Lit[137] bp 80-85°C/0.2 mm Hg) to obtain the title compound as a colourless oil (7.9 g, 71%).

υ_{max} (DCM film)/ cm^{-1} 1799 (CO, acid chloride), 1603 (CH, aryl); δ_H ($CDCl_3$; 250 MHz) 2.11 (2H, tt, *J*=7.2, 7.2 Hz, $CH_2CH_2CH_2$), 2.76 (2H, t, *J*=7.3 Hz, $CH_2CH_2CH_2$), 2.97 (2H, t, *J*=7.2 Hz, $CH_2CH_2CH_2$), 7.23-7.42 (5H, m, Ph); δ_C ($CDCl_3$; 63 MHz) 26.7 (CH_2), 34.4 (CH_2), 46.4 (CH_2), 126.5 (CH_{arom}), 128.5 (CH_{arom} x 2), 128.7 (CH_{arom} x 2), 140.4 (C_{arom}), 173.8 (*C*O);

8.4.2 General procedure for the preparation of N-acyl benzotriazoles

To a solution of benzotriazole (1eq.) in anhydrous dichloromethane was added triethylamine (1.2eq.), followed by the appropriate acid chloride (1.1eq.). The reaction was stirred at room temperature for 0.5 h. After this time, the reaction mixture was washed with 1M HCl and saturated $NaHCO_3$. The organic layers were dried over magnesium sulfate and concentrated to obtain the product.

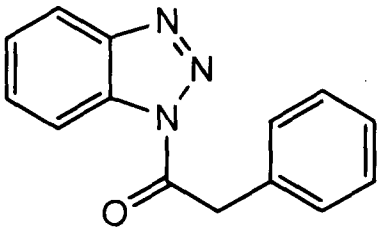
8.4.3 Benzotriazol-1-yl-phenyl-methanone 270[110]



General procedure 8.4.2 was followed with benzotriazole **224** (1.0 g, 8.6 mmol), benzoyl chloride (1.1 mL, 9.5 mmol), triethylamine (1.44 mL, 10.3 mmol) and dichloromethane (50 mL). Recrystallisation [Hexane/ethyl acetate (9:1)] afforded the title compound as a colourless solid (56%).

R_f 0.78 [ethyl acetate/hexane (3:7)]; Mp 106-108 °C Lit[110] Mp 112-113 °C; υ_{max} (nujol mull)/ cm^{-1}, 1710 (CO), 1597 (C=C, arom); δ_H ($CDCl_3$; 250 MHz) 7.67-7.91 (5H, m, Ph), 8.37 (1H, ddd, *J*=8.2, 8.1, 0.9 Hz, CH_{bt}), 8.44 (1H, ddd, *J*=8.3, 8.3, 1.0 Hz, CH_{bt}), 8.58 (1H, ddd, *J*=7.6, 0.8, 0.8 Hz, CH_{bt}), 8.76 (1H, ddd, *J*=8.3, 0.9, 0.9 Hz, CH_{bt}); δ_C ($CDCl_3$; 63 MHz) 115.2 (*C*H_{bt}), 120.6 (*C*H_{bt}), 126.7 (*C*H_{bt}), 128.8 (*C*H_{arom} x 2), 130.8 (*C*H_{bt}), 131.8 (C_{bt}), 132.1 (*C*H_{arom} x 2), 132.7 (C_{bt}), 134.06 (*C*H_{arom}), 146.1 (C_{arom}), 167.1 (*C*=$O_{N\text{-}acyl}$); FAB-MS, Found: MH^+ 224.0819, $C_{13}H_{10}N_3O$ requires MH^+ 224.0824.

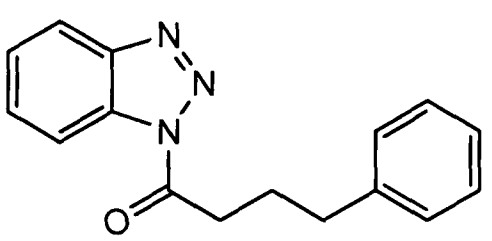
8.4.4 1-Benzotriazol-1-yl-2-phenyl-ethanone 271[135]



General procedure 8.4.2 was followed with benzotriazole **224** (1.54 g, 12.9 mmol), phenylacetyl chloride (1.87 mL, 14.2 mmol), triethylamine (2.16 mL, 15.5 mmol) and dichloromethane (75 mL). Recrystallisation [Hexane/diethyl ether (9:1)] afforded the title compound as a colourless solid (2.2 g, 73%).

R_f 0.73 [ethyl acetate/hexane (3:7)]; Mp 67-70 °C, Lit[135] Mp 66-67 °C; υ_{max} (nujol mull)/ cm^{-1}, 1733 (CO, *N*-acyl), 1593 (C=C, arom); δ_H ($CDCl_3$; 250 MHz) 4.96 (2H, s, C*H*$_2$), 7.56-7.71 (5H, m, Ph), 7.73 (1H, ddd, *J*=8.3, 8.1, 0.9 Hz, C*H*$_{bt}$), 7.87 (1H, ddd, *J*=7.1, 7.0, 1.0 Hz, C*H*$_{bt}$), 8.42 (1H, ddd, *J*=8.2, 1.0, 1.0 Hz, C*H*$_{bt}$), 8.50 (1H, ddd, *J*=8.8, 0.9, 0.9 Hz, C*H*$_{bt}$); δ_C ($CDCl_3$; 63 MHz) 41.9 (*C*H$_2$), 114.4 (*C*H$_{bt}$), 120.1 (*C*H$_{bt}$), 126.1 (*C*H$_{bt}$), 127.5 (*C*H$_{bt}$), 128.7 (*C*H$_{arom}$ x 2), 129.7 (*C*H$_{arom}$ x 2), 130.4 (*C*H$_{arom}$), 131.1 (*C*$_{bt}$), 132.4 (*C*$_{bt}$), 146.2 (*C*$_{arom}$), 170.1 (*C*=O$_{N\text{-acyl}}$); FAB-MS, Found: MH^+ 238.0984, $C_{14}H_{12}N_3O$ requires MH^+ 238.0980.

8.4.5 1-Benzotriazol-1-yl-4-phenyl-butan-1-one 272



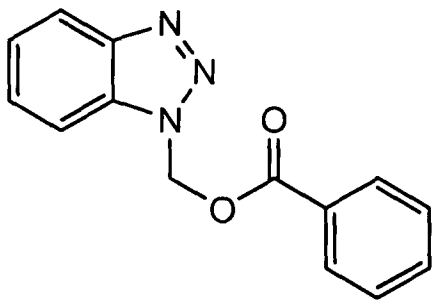
General procedure 8.4.2 was followed with benzotriazole **224** (1.54 g, 12.9 mmol), 4-phenylbutyryl chloride (2.35 mL, 14.2 mmol), triethylamine (2.16 mL, 15.5 mmol) and dichloromethane (75 mL). Recrystallisation [Hexane/ethyl acetate (9:1)] afforded the title compound as an off-white solid (2.5 g, 71%).

R_f 0.73 [ethyl acetate/hexane (3:7)]; Mp 67-70 °C; υ_{max} (nujol mull)/ cm^{-1}, 1741 (CO, *N*-acyl), 1593 (C=C, arom); δ_H ($CDCl_3$; 250 MHz) 2.27 (2H, tt, *J*=7.6, 7.5 Hz, $CH_2CH_2CH_2$), 2.89 (2H, t, *J*=7.2 Hz, $CH_2CH_2CH_2$), 3.52 (2H, t, *J*=7.4 Hz, C*H*2CH2CH2), 7.25-7.36 (5H, m, Ph), 7.56 (1H, ddd, *J*=7.2, 7.2, 1.0 Hz, C*H*$_{bt}$), 7.71 (1H, ddd, *J*=8.0, 8.0, 1.0 Hz, C*H*$_{bt}$), 8.17 (1H, ddd, *J*=7.4, 1.0, 1.0 Hz, C*H*$_{bt}$), 8.33 (1H, ddd, *J*=8.2, 0.9, 0.9 Hz, C*H*$_{bt}$); δ_C ($CDCl_3$; 63 MHz) 25.7 (*C*H$_2$), 34.7 (*C*H$_2$), 34.9 (*C*H$_2$), 114.3 (*C*H$_{bt}$), 120.0 (*C*H$_{bt}$), 125.9 (*C*H$_{arom}$), 126.0 (*C*H$_{bt}$), 128.4 (*C*H$_{arom}$ x 2), 128.4 (*C*H$_{arom}$ x 2), 130.2 (*C*H$_{bt}$), 130.9 (*C*$_{bt}$), 140.8 (*C*$_{bt}$), 146.0 (*C*$_{arom}$), 172.2 (*C*=O$_{N\text{-acyl}}$); FAB-MS, Found: MH^+ 266.1300, $C_{16}H_{16}N_3O$ requires MH^+ 266.1293.

8.4.6 General procedure for the preparation of benzotriazole hemiaminals

To a mixture of *N*-acyl benzotriazole (1eq.) and desired aldehyde (1.1eq.) in acetonitrile was added triethylamine (1.2eq.) with stirring. The reaction was stirred at room temperature. After TLC had indicated the reaction had gone to completion, the reaction mixture was evaporated, then diluted with DCM, washed with 1 M HCl, basified with $NaHCO_3$ and dried over $MgSO_4$ to give the crude product.

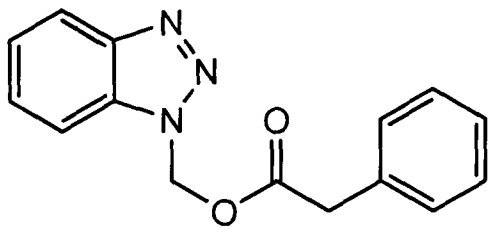
8.4.7 Benzoic acid benzotriazole-1-ylmethyl hemiaminal 273[113,136]



General procedure 8.4.6 was followed with **270** (0.40 g, 1.8 mmol), formaldehyde (37% in $MeOH/H_2O$, 0.05 mL, 2 mmol) and triethylamine (0.30 mL, 2.1 mmol) in acetonitrile (8 mL) for 60 min. The crude product was purified on a 40S Biotage system [(3:7) ethyl acetate/ hexane] to give the title compound as a colourless solid (0.30 g, 66%).

R_f 0.51 [ethyl acetate/hexane (3:7)]; Mp 84-87 °C Lit[136] Mp 91-92 °C; υ_{max} (nujol mull)/ cm^{-1}, 1733 (CO, ester), 1599 (C=C, arom); δ_H ($CDCl_3$; 250 MHz) 7.03 (2H, s, C*H*$_2$), 7.57-7.77 (5H, m, Ph), 8.07 (1H, ddd, *J*=8.3, 0.9, 0.9 Hz, C*H*$_{bt}$), 8.20 (1H, ddd, *J*=8.5, 8.3, 1.3 Hz, C*H*$_{bt}$), 8.22 (1H, ddd, *J*=8.5, 7.6, 0.7 Hz, C*H*$_{bt}$), 8.76 (1H, ddd, *J*=8.3, 0.9, 0.9 Hz, C*H*$_{bt}$); δ_C ($CDCl_3$; 63 MHz) 68.2 (*C*H$_2$), 110.0 (*C*H$_{bt}$), 119.9 (*C*H$_{bt}$), 124.5 (*C*H$_{bt}$), 128.2 (*C*$_{bt}$), 128.4 (*C*H$_{arom}$ x 2), 128.4 (*C*H$_{bt}$), 129.9 (*C*H$_{arom}$ x 2), 132.7 (*C*$_{bt}$), 133.8 (*C*H$_{arom}$), 146.0 (*C*$_{arom}$), 165.4 (*C*=O$_{ester}$); FAB-MS, Found: MH^+ 254.0934, $C_{14}H_{12}N_3O_2$ requires MH^+ 254.0929.

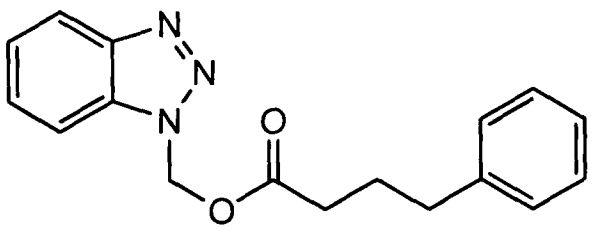
8.4.8 *Phenyl acetic acid benzotriazole-1-ylmethyl hemiaminal 274*



General procedure 8.4.6 was followed with **271** (0.47 g, 2.0 mmol), formaldehyde (37% in $MeOH/H_2O$, 0.06 mL, 2 mmol) and triethylamine (0.33 mL, 2.4 mmol) in acetonitrile (8 mL) for 16 h. The crude product was purified on a 40S Biotage system [(2:8) ethyl acetate/ hexane] to give the title compound as a colourless solid (0.24 g, 45%).

R_f 0.46 [ethyl acetate/hexane (3:7)]; Mp 88-90 °C; υ_{max} (nujol mull)/cm^{-1}, 1752 (CO, ester), 1600 (C=C, arom); δ_H ($CDCl_3$; 250 MHz) 3.90 (2H, s, CH_2Ph), 6.80 (2H, s, CH_2O), 7.45-7.56 (5H, m, Ph), 7.70 (1H, ddd, *J*=8.2, 8.2, 1.1 Hz, CH_{bt}), 7.79 (1H, ddd, *J*=8.2, 8.2, 1.1 Hz, CH_{bt}), 7.95 (1H, ddd, *J*=8.3, 0.8, 0.8 Hz, CH_{bt}), 8.34 (1H, ddd, *J*=8.3, 0.8, 0.8 Hz, CH_{bt}); δ_C ($CDCl_3$; 63 MHz), 40.6 (CH_2), 68.0 (CH_2), 109.9 (CH_{bt}), 119.9 (CH_{bt}), 124.4 (CH_{bt}), 127.3 (CH_{bt}), 128.3 (CH_{arom}), 128.6 (CH_{arom} x 2), 129.1 (CH_{arom} x 2), 132.5 (C_{bt}), 132.6 (C_{bt}), 146.0 (C_{arom}), 170.7 ($C{=}O_{ester}$); *m/z* (ES-MS; +ve), 268 (30%, MH^+), 290 (100%, MNa^+); FAB-MS, Found: MH^+ 268.1090, $C_{15}H_{13}N_3O_2$ requires MH^+ 268.1086.

8.4.9 *4-Phenylbutyric acid benzotriazole-1-ylmethyl hemiaminal 275*

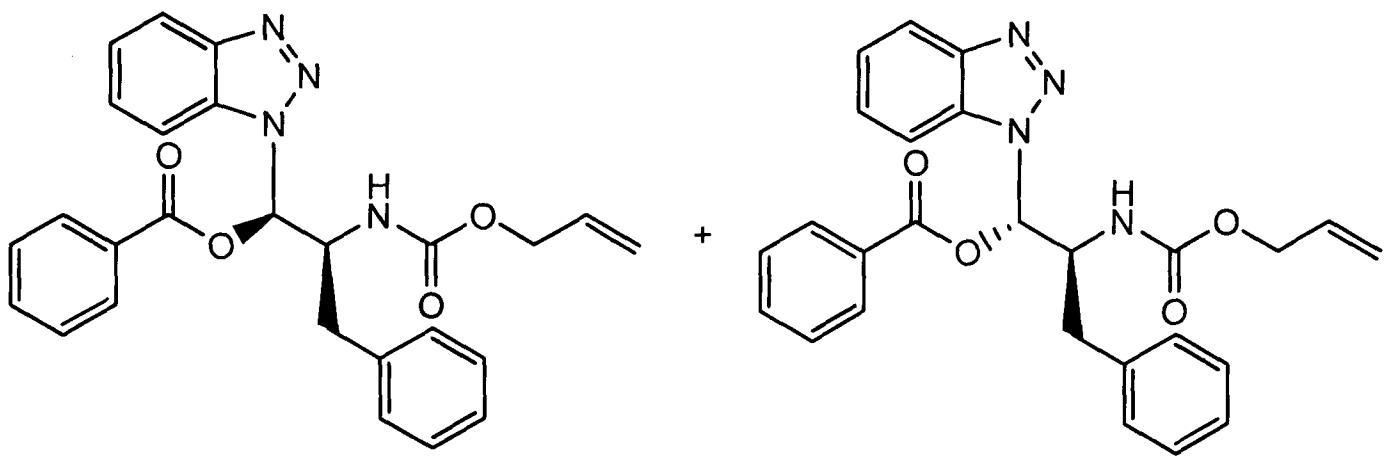


General procedure 8.4.6 was followed with **272** (0.47 g, 1.7 mmol), formaldehyde (37% in $MeOH/H_2O$, 0.05 mL, 2 mmol) and triethylamine (0.28 mL, 2.0 mmol) in acetonitrile (8 mL) for 16 h. The crude product was purified on silica [(3:7) ethyl acetate/ hexane] to give the title compound as a colourless oil (0.32 g, 63%).

R_f 0.55 [ethyl acetate/hexane (3:7)]; υ_{max} (DCM film)/ cm^{-1}, 1749 (CO, ester), 1602 (C=C, arom); δ_H ($CDCl_3$; 250 MHz) 1.92-2.01 (2H, tt, *J*= 7.4, 7.4 Hz, $CH_2CH_2CH_2$),

2.44 (2H, t, *J*=7.4 Hz, C*H*$_2$CH$_2$CH$_2$), 2.63 (2H, t, *J*=7.4 Hz, CH$_2$CH$_2$C*H*$_2$), 6.61 (2H, s, C*H*$_2$O), 7.11-7.529 (5H, m, Ph), 7.48 (1H, ddd, *J*=8.3, 8.3, 1.1 Hz, C*H*$_{bt}$), 7.61 (1H, ddd, *J*=8.3, 8.3, 1.0 Hz, C*H*$_{bt}$), 7.81 (1H, ddd, *J*=8.3, 0.9, 0.9 Hz, C*H*$_{bt}$), 8.14 (1H, ddd, *J*=8.3, 0.9, 0.9 Hz, C*H*$_{bt}$); δ_C ($CDCl_3$; 63 MHz), 25.9 (*C*H$_2$), 32.9 (*C*H$_2$), 34.7 (*C*H$_2$), 67.5 (*C*H$_2$), 109.9 (*C*H$_{bt}$), 119.9 (*C*H$_{bt}$), 124.4 (*C*H$_{bt}$), 125.9 (*C*H$_{bt}$), 128.3 (*C*H$_{arom}$ x 5), 132.6 (*C*$_{bt}$), 140.7 (*C*$_{bt}$), 145.9 (*C*$_{arom}$), 172.4 (*C*=O$_{ester}$); *m/z* (ES-MS; +ve), 296 (15%, MH$^+$), 318 (100%, MNa$^+$); FAB-MS, Found: MH$^+$ 296.1392, $C_{17}H_{17}N_3O_2$ requires MH$^+$ 296.1399.

8.4.10 (1R,2S)- and (1S,2S)-Benzoic acid 2-allyloxycarbonylamino-1-benzotriazol-1-yl-3-phenyl-propyl hemiaminals 278a/b

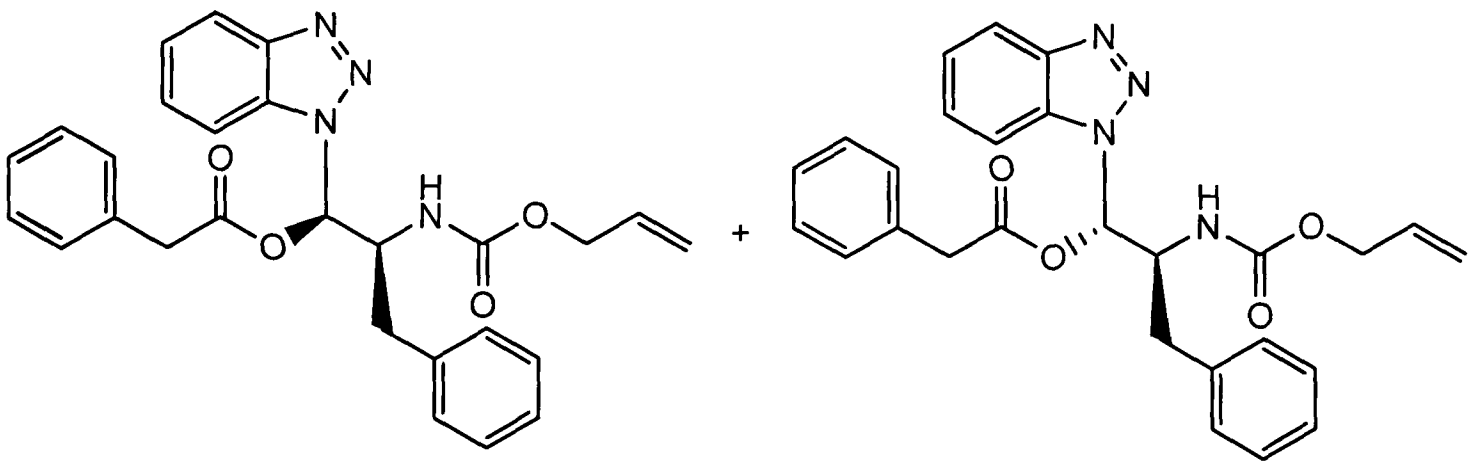


General procedure 8.4.6 was followed using **270** (0.40 g, 1.8 mmol), **171** (0.45 g, 2.0 mmol) and triethylamine (0.30 mL, 2.1 mmol) in acetonitrile/ dichloromethane (90:10) (8 mL). The crude product was purified on a 40S Biotage system [(2:8) ethyl acetate/ hexane] to give the title compound as a diastereomeric mixture (0.45 g, 55%).

R_f 0.45 [ethyl acetate/hexane (3:7)]; Mp 107-109.5 °C; υ_{max} (film)/ cm^{-1}, 1730 (CO, ester and urethane), 1648 (CO, amide II),1601 (C=C, arom); δ_H ($CDCl_3$; 250 MHz) 2.80 (1H, dd, *J*=13.5, 8.3 Hz, C*H*$_a$CH$_b$Ph, one diastereomer), 2.83 (2H, d, *J*=5.0 Hz, CH$_a$C*H*$_b$Ph and C*H*$_a$CH$_b$Ph, two diastereomers), 3.20 (1H, dd, *J*=14.1, 7.6 Hz, CH$_a$C*H*$_b$Ph, one diastereomer), 4.49 (2H, d, *J*=5.2 Hz, C*H*$_2$O, one diastereomer), 4.61 (2H, d, *J*=5.5 Hz, C*H*$_2$O, one diastereomer), 5.06-5.31 (2 x 1H and 2 x 1H and 2 x 1H, overlapping m, C*H*CH$_2$, two diasteromers and CH=C*H*$_{cis}$CH$_{trans}$, two diastereomers and CH=CH$_{cis}$ C*H*$_{trans}$, two diastereomers), 5.73 (2 x 1H, overlapping ddd, *J*=22.4, 10.5, 5.3 Hz, C*H*=CH$_2$, 2 diastereomers), 6.45 (2 x 1H, N*H*, two

diastereomers), 7.05 (2 x 1H, m, OC*H*CH), 7.19-7.33 (2 x 10H, m, Ph, two diastereomers), 7.44-7.50 (2 x 1H, m, CH_{bt}, two diastereomers), 7.59-7.66 (2 x 1H, m, CH_{bt}, two diastereomers), 7.98-8.16 (2 x 1H and 2 x 1H, m, CH_{bt}, two diastereomers); δ_C ($CDCl_3$; 63 MHz) 35.3 (*C*H_2Ph, two diastereomers), 53.1 (*C*HCH_2, major diastereomer), 54.5 (*C*HCH_2, minor diastereomer), 65.0 (*C*H_2O, two diastereomers), 76.4 (*C*HCH, minor diastereomer), 79.3 (*C*HCH, major diastereomer), 109.8 (*C*H_{bt}, two diastereomers), 117.1 (*C*H_2=CH, two diastereomers), 119.5 (*C*H_{bt}, two diastereomers), 124.0 (*C*H_{bt}, two diastereomers), 126.4 (*C*H_{arom}, two diastereomers), 127.5 (C_{bt}, two diastereomers), 127.9 (*C*H_{bt}, two diastereomers), 128.1 (*C*H_{arom} x 2, two diastereomers), 128.4 (*C*H_{arom} x 2, two diastereomers), 128.6 (*C*H_{arom}, two diastereomers), 129.5 (*C*H_{arom} x 2, two diastereomers), 131.6 (*C*H_{arom} x 2, two diastereomers), 132.0 (C_{bt}, two diastereomers), 133.3 (*C*H=CH_2, two diastereomers), 134.9 (C_{arom}, two diastereomers), 145.2 (C_{arom}, two diastereomers), 155.2 (*C*=$O_{urethane}$, two diastereomers), 164.3 (*C*=O_{ester}, two diastereomers); FAB-MS, Found: MH^+ 457.1882, $C_{26}H_{25}N_4O_4$ requires MH^+ 457.1875.

8.4.11 *(1R,2S)- and (1S,2S)- Phenylacetic acid 2-allyloxycarbonylamino-1-benzotriazol-1-yl-3-phenyl-propyl hemiaminals 279a/b*

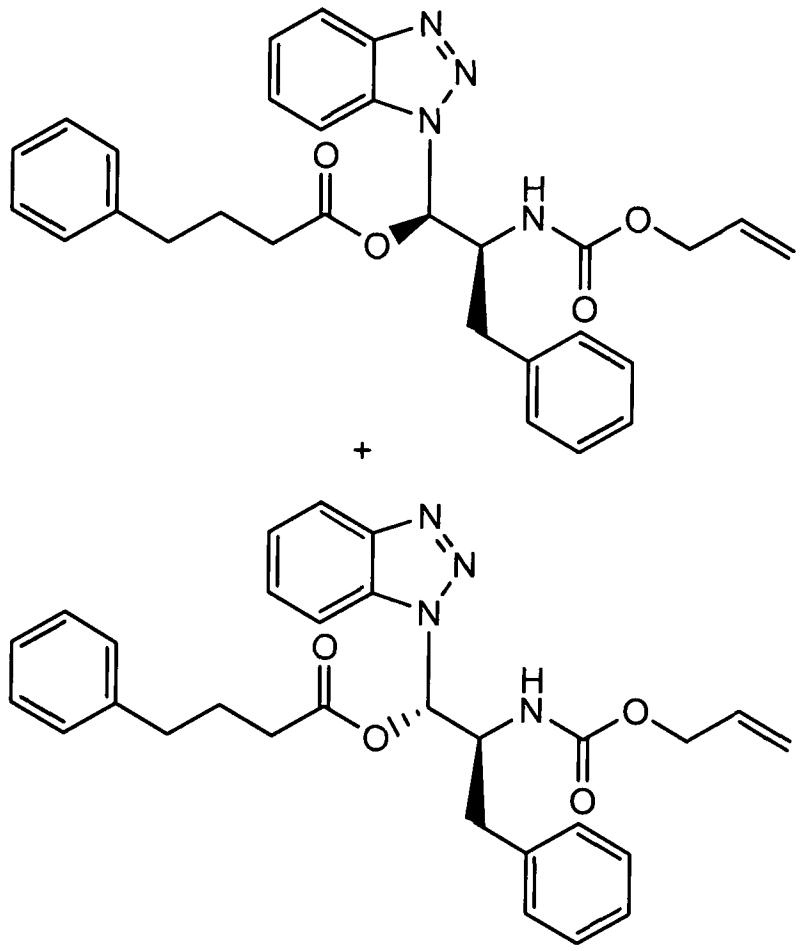


General procedure 8.4.6 was followed using **271** (0.40 g, 1.7 mmol), **171** (0.43 g, 1.9 mmol) and triethylamine (0.35 mL, 2.5 mmol) in dichloromethane (10 mL). The crude product was purified on silica [(19:1) dichloromethane/ diethyl ether] to give the title compounds as a diastereomeric mixture (0.25 g, 32%).

R_f 0.35 [ethyl acetate/hexane (3:7)]; υ_{max} (film)/ cm^{-1}, 1754 (CO, ester), 1722 (CO, urethane),1647 (CO, amide II), 1603 (C=C, arom); δ_H ($CDCl_3$; 250 MHz) 2.62 (1H, dd, *J*=13.9, 7.9 Hz, C*H*$_a$CH$_b$Ph, one diastereomer), 2.76 (2H, d, *J*=4.8 Hz, CH$_a$C*H*$_b$Ph and C*H*$_a$CH$_b$Ph, two diastereomers), 2.91 (1H, dd, *J*=13.8, 7.6 Hz, CH$_a$C*H*$_b$Ph, one diastereomer), 3.56 (2H, s, COC*H*$_2$Ph, one diastereomer), 3.57 (2H, s, COC*H*$_2$Ph, one diastereomer), 4.38 (2H, d, *J*=5.0 Hz, C*H*$_2$O, one diastereomer), 4.45 (2H, d, *J*=4.5 Hz, C*H*$_2$O, one diastereomer), 4.83 (2 x 1H, m, C*H*CH$_2$, two diastereomers), 5.07 (2 x 1H, overlapping dd, *J*=10.4, 1.3 Hz, CH=C*H*$_{cis}$CH$_{trans}$, two diastereomers), 5.18 (2 x 1H, overlapping dd, *J*=15.9, 1.5 Hz, CH=CH$_{cis}$ C*H*$_{trans}$, two diastereomers), 5.65-5.85 (2 x 1H, overlapping ddt, *J*=15.8, 10.5, 5.3 Hz, C*H*=CH$_2$, two diastereomers), 5.95 (2 x 1H, N*H*, two diastereomer), 6.83 (2 x 1H, m, OC*H*CH, two diastereomers), 6.98-7.19 (2 x 10H, m, Ph, two diastereomers), 7.30-7.65 (6 x 1H, m, C*H*$_{bt}$, two diastereomers), 8.00 (1H, ddd, *J*=8.2, 1.0, 1.0 Hz, C*H*$_{bt}$, one diastereomer), 8.01 (1H, ddd, *J*=8.2, 1.0, 1.0 Hz, C*H*$_{bt}$, one diastereomer); δ_C ($CDCl_3$; 63 MHz) 35.9 (*C*H$_2$Ph, minor diastereomer), 37.2 (*C*H$_2$Ph, major diasteromer), 40.4 (*C*H$_2$Ph, minor diastereomer), 40.6 (*C*H$_2$Ph, major diasteromer), 54.1 (*C*HCH$_2$, minor diastereomer), 54.7 (*C*HCH$_2$, major diastereomer), 65.6 (O*C*H$_2$, minor diastereomer), 65.7 (O*C*H$_2$, major diastereomer), 77.5 (*C*HCH, minor diastereomer), 79.0 (*C*HCH, major diastereomer), 110.1 (*C*H$_{bt}$, major diastereomer), 110.3 (*C*H$_{bt}$, minor diastereomer), 117.4 (*C*H$_2$=CH, minor diastereomer), 117.8 (*C*H$_2$=CH, major diastereomer), 119.8 (*C*H$_{bt}$, minor diastereomer), 120.0 (*C*H$_{bt}$, major diastereomer), 124.5 (*C*H$_{bt}$, major diastereomer), 124.6 (*C*H$_{bt}$, minor diastereomer), 126.9 (*C*H$_{bt}$, major diastereomer), 127.4 (*C*H$_{bt}$, minor diastereomer), 128.2 (*C*H$_{arom}$ x 2, major diastereomer), 128.4 (*C*H$_{arom}$ x 2, minor diastereomer), 128.5 (*C*H$_{arom}$ x 2, one diastereomer), 128.6 (*C*H$_{arom}$ x 2, one diastereomer), 128.8 (*C*H$_{arom}$ x 4, one diastereomer), 129.1 (*C*H$_{arom}$ x 4, one diastereomer), 129.1 (*C*H$_{arom}$ x 2, one diastereomer), 129.2 (*C*H$_{arom}$ x 2, one diastereomer), 132.3 (*C*H=CH$_2$, minor diastereomer), 132.4 (*C*H=CH$_2$, major diastereomer), 132.6 (*C*$_{bt}$ x 2, major diastereomer), 133.2 (*C*$_{bt}$ x 2, minor diastereomer), 135.6 (*C*$_{arom}$, minor diastereomer), 135.8 (*C*$_{arom}$, major diastereomer), 145.0 (*C*$_{arom}$, minor diastereomer), 145.6 (*C*$_{arom}$, major diastereomer), 155.4 (*C*=O$_{urethane}$, major diastereomer), 155.9 (*C*=O$_{urethane}$, minor diastereomer), 169.8 (*C*=O$_{ester}$, major diastereomer), 170.0

(C=O_{ester} minor diastereomer); FAB-MS, Found: MH^+ 471.2031, $C_{27}H_{27}N_4O_4$ requires MH^+ 471.2032.

8.4.12 *(1R,2S)- and (1S,2S)-1-(4-Phenylbutyric acid) 2-allyloxycarbonylamino-1-benzotriazol-1-yl-3-phenyl-propyl hemiaminal 280a/b*



General procedure 8.4.6 was followed using **272** (0.40 g, 1.5 mmol), **171** (0.38 g, 1.7 mmol) and triethylamine (0.31 mL, 2.2 mmol) in dichloromethane (8 mL). The crude product was purified on silica [(19:1) dichloromethane/diethyl ether] to give the title compound as diastereomeric mixture (0.33 g, 44%).

R_f 0.30 [ethyl acetate/hexane (3:7)]; υ_{max} (film)/ cm^{-1}, 1752 (CO, ester), 1722 (CO, urethane), 1647 (CO, amide II), 1603 (C=C, arom); δ_H ($CDCl_3$; 250 MHz) 1.73-1.79 (2H x 2, overlapping tt, J=7.2, 7.2 Hz, $CH_2CH_2CH_2$, two diastereomers), 2.17-2.25 (2H x 2, overlapping t, J= 7.4 Hz, $CH_2CH_2CH_2$, two diastereomers), 2.35-2.46 (2H x 2, overlapping t, J= 7.4 Hz, $CH_2CH_2CH_2$, two diastereomers), 2.50-2.62 (1H, dd, J=13.9, 8.3 Hz, CH_aCH_bPh, one diastereomer), 2.76 (2H, d, J=5.8 Hz, CH_aCH_bPh and CH_aCH_bPh, two diastereomers), 2.86-3.00 (1H, dd, J=14.1, 7.6 Hz, CH_aCH_bPh, one diastereomer), 4.33 (2H, d, J=5.4 Hz, CH_2O, one diastereomer), 4.38 (2H, J=5.5 Hz, CH_2O, one diastereomer), 4.32-4.37 (2 x 1H, overlapping m, $CHCH_2$, two diastereomers), 5.01 (2 x 1H, overlapping dd, J=11.0, 1.3 Hz, $CH=CH_{cis}CH_{trans}$, two

diastereomers), 5.05 (2 x 1H, overlapping dd, *J*=22.0, 1.4 Hz, CH=CH$_{cis}$C*H*$_{trans}$, two diastereomers), 5.57-5.80 (2 x 1H, overlapping ddt, *J*=22.5, 11.3, 5.4 Hz, C*H*=CH$_2$, two diastereomers), 5.94 (2 x 1H, N*H*, two diastereomers), 6.83 (2 x 1H, overlapping m, C*H*CH, two diastereomers), 6.98-7.23 (2 x 10H, m, Ph, two diastereomers), 7.30-7.65 (6 x 1H, m, C*H*$_{bt}$, two diastereomers), 8.00 (1H, ddd, *J*=8.2, 1.0, 1.0 Hz, C*H*$_{bt}$, one diastereomer), 8.01 (1H, ddd, *J*=8.2, 1.0, 1.0 Hz, C*H*$_{bt}$, one diastereomer); δ_C ($CDCl_3$; 63 MHz) 25.7 (*C*H$_2$, minor diastereomer), 25.9 (*C*H$_2$, major diastereomer), 32.8 (*C*H$_2$, minor diastereomers), 34.6 (*C*H$_2$, major diastereomers), 35.9 (*C*H$_2$Ph, major diastereomer), 37.5 (*C*H$_2$Ph, minor diastereomer), 53.9 (*C*H$_2$Ph, major diastereomer), 54.7 (*C*H$_2$Ph, minor diastereomer), 65.5 (O*C*H$_2$, minor diastereomer), 65.7 (O*C*H$_2$, major diastereomer), 76.8 (*C*HCH, minor diastereomer), 78.7 (*C*HCH, major diastereomer), 110.2 (*C*H$_{bt}$, major diastereomer), 110.3 (*C*H$_{bt}$, minor diastereomer), 117.4 (*C*H$_2$=CH, minor diastereomer), 117.7 (*C*H$_2$=CH, major diastereomer), 119.8 (*C*H$_{bt}$, minor diastereomer), 120.1 (*C*H$_{bt}$, major diastereomer), 124.5 (*C*H$_{bt}$, major diastereomer) , 124.6 (*C*H$_{bt}$, minor diastereomer), 125.9 (*C*H$_{bt}$, major diastereomer), 126.9 (*C*H$_{bt}$, minor diastereomer), 128.3 (*C*H$_{arom}$ x 4, one diastereomer), 128.3 (*C*H$_{arom}$ x 4, one diastereomer), 128.6 (*C*H$_{arom}$ x 4, one diastereomer), 128.8 (*C*H$_{arom}$ x 4, one diastereomer), 129.1 (*C*H$_{arom}$ x 2, two diastereomers), 132.2 (*C*H=CH$_2$, minor diastereomer), 132.5 (*C*H=CH$_2$, major diastereomer), 132.6 (*C*$_{bt}$, major diastereomer), 133.2 (*C*$_{bt}$, minor diastereomer), 135.6 (*C*$_{arom}$, major diastereomer), 135.8 (*C*$_{arom}$, minor diastereomer), 140.6 (*C*$_{bt}$, two diastereomers), 145.0 (*C*$_{arom}$, minor diastereomer), 145.7 (*C*$_{arom}$, major diastereomer), 155.5 (*C*=O$_{urethane}$, major diastereomer), 155.9 (*C*=O$_{urethane}$, minor diastereomer), 171.7 (*C*=O$_{ester}$, two diastereomers); *m/z* (ES-MS; +ve), 521 (100%, MNa^+); FAB-MS, Found: MH^+ 499.2339, $C_{29}H_{31}N_4O_4$ requires MH^+ 499.2345.

8.5 Enzymatic studies on benzotriazole substrates

8.5.1 Screening of enzymes for hydrolysis of benzotriazole hemiaminals

Hydrolysis experiments were carried out in glass sample tubes (2 mL) with plastic stoppers. To the enzyme (~0.8 mg) was added 1 mL of stock substrate solution (made up with potassium phosphate buffer pH 7.4/acetonitrile (9:1), to give ~0.3 μmol per reaction for **273-275** and 0.9 μmol for **278a/b-280a/b**. The solutions were rotated for 16 h at room temperature on a 360° blood rotator. An aliquot was removed for analysis by RP-HPLC. Control experiments contained all reagents except the enzyme.

HPLC conditions: Using equipment according to Section 8.2.9 with an isocratic method eluting with $H_2O/CH_3CN/TFA$ (7:3:0.01).

Specificity of HPLC method:

Analyte	R_t (min)
Benzotriazole	4.4
Benzoic acid	6.7
Phenylacetic acid	6.3
4-phenylbutyric acid	15.1
171	9.6

Table 8: *Specificity of HPLC method.*

HPLC conditions for determination of diastereoselectivity: Using equipment according to Section 8.2.9 with an isocratic method eluting with $H_2O/CH_3CN/TFA$ (5:5:0.01).

Specificity of HPLC method for determination of diastereoselectivity:

Analyte	R_t (min)
278 (major diastereomer)	20.6
278 (minor diastereomer)	22.5
279 (major diastereomer)	20.8

279 (minor diastereomer)	22.2
280 (major diastereomer)	36.9
280 (minor diastereomer)	41.1

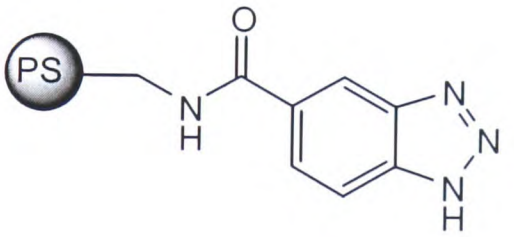
Table 9: *Specificity of HPLC method for determination of diastereoselectivity.*

8.6 Solid phase synthesis of linkers

8.6.1 General procedure for the preparation of benzotriazole resins[103]

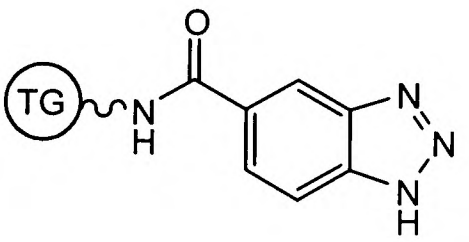
Benzotriazole-5-carboxylic acid **207** (10eq.) and HOBt (10eq.) were suspended in dimethylformamide. Diisopropylcarbodiimide (5eq.) was added and the mixture stirred at room temperature for 1 h. Appropriate resin (1eq.) was added and the mixture shaken at room temperature for 16 h. After this time, the Kaiser test 8.2.5.1 was carried out to confirm coupling, and the resin was filtered and washed according to the standard protocol 8.2.3.

8.6.2 1H-Benzotriazole-5-carboxylic acid-methylamino polystyrene 218



General procedure 8.6.1 was followed with benzotriazole-5-carboxylic acid **207** (4.11 g, 25.2 mmol), HOBt (3.40 g, 25.2 mmol), diisopropylcarbodiimide (1.97 mL, 12.6 mmol) and amino-methylated polystyrene HL **214**, 100-200 mesh (1.23 mmol g^{-1}, 2.05 g, 2.5 mmol) in dimethylformamide (45 mL). The title compound was obtained as brown beads. Kaiser (8.2.5.1) test confirmed quantitative coupling. υ_{max} (DCM swell)/ cm^{-1}, 1652 (CO, amide I), 1540 (CO, amide II); δ_H (MAS-NMR; $CDCl_3$; 600 MHz) 7.84 (broad s, CH_{bt}).

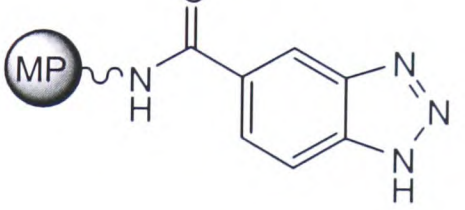
8.6.3 1H-Benzotriazole-5-carboxylic acid-amino tentagel 217



General procedure 8.6.1 was followed with benzotriazole-5-carboxylic acid **207** (0.52 g, 3.2 mmol), HOBt (0.43 g, 3.2 mmol), diisopropylcarbodiimide (0.25 mL, 1.6 mmol) and amino-TentaGel HL **213** (0.45 mmol g^{-1}, 0.71 g, 0.32 mmol) in DMF (45 mL). The title compound was obtained as a brown gel. Kaiser 8.2.5.1 test confirmed quantitative coupling.

υ_{max} (DCM swell)/ cm^{-1} 1650 (CO, amide I), 1540 (CO, amide II); δ_C ($CDCl_3$; 63 MHz) 167.0 (*C*O), 132.0 (*C*H_{bt}), 124.8 (*C*H_{bt}), 115.5 (*C*H_{bt}).

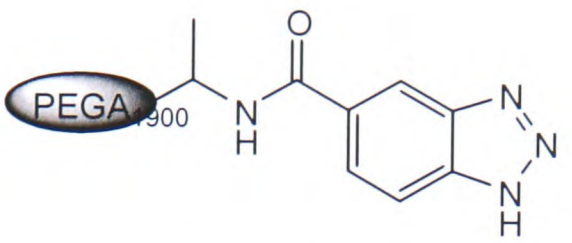
8.6.4 1H-Benzotriazole-5-carboxylic acid-aminomethyl polystyrene MP 219



General procedure 8.6.1 was followed with benzotriazole-5-carboxylic acid **207** (3.88 g, 23.8 mmol), HOBt (3.22 g, 23.8 mmol), diisopropylcarbodiimide (1.86 mL, 11.9 mmol) and aminomethyl polystyrene MP **215**, particle size=50-70 μm, pore size=1000 Å (1.19 mmol g^{-1}, 2.00 g, 2.4 mmol). The title compound was obtained as non-swelling brown beads. Kaiser 8.2.5.1 test confirmed quantitative coupling.

υ_{max} (KBr disc)/ cm^{-1}, 1645 (CO, amide I).

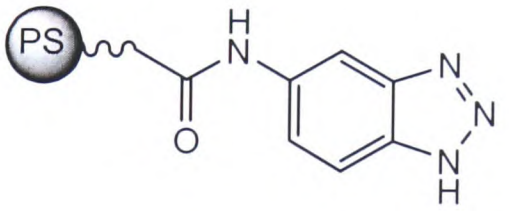
8.6.5 1H-Benzotriazole-5-carboxylic acid-amino-PEGA$_{1900}$ 220



General procedure 8.6.1 was followed with benzotriazole-5-carboxylic acid **207** (0.97 g, 6.0 mmol), HOBt (0.81 g, 6.0 mmol), diisopropylcarbodiimide (0.47 mL, 3.0 mmol) and PEGA$_{1900}$ **216**, particle size=300-500 μm, (0.2 mmol g^{-1}, 3 g, 0.6 mmol) in dimethylformamide (35 mL). The coupled resin was obtained as a pale brown gel and stored in DCM until required for further elaboration. Kaiser 8.2.5.1 test confirmed quantitative coupling.

υ_{max} (KBr disc)/ cm^{-1}, 1672 (CO, amide I), 1549 (CO, amide II); δ_H (MAS-NMR; $CDCl_3$; 600 MHz) 7.60-8.50 (broad m, CH_{bt}).

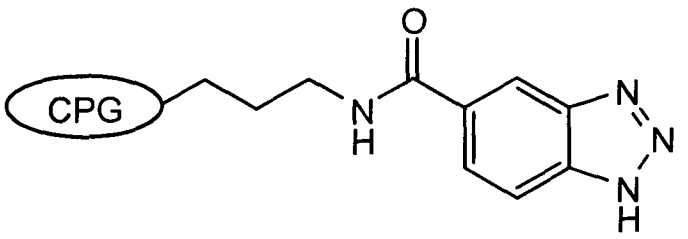
8.6.6 1H-Benzotriazole-5-amino-carboxypolystyrene 254



Carboxy-polystyrene HL, 100-200mesh (1.24 mmol g^{-1}, 1.95 g, 2.4 mmol) and HOBt (3.27 g, 24.2 mmol) were suspended in dimethylformamide. Diisopropylcarbodiimide (1.53 g, 12.1 mmol) was added and the mixture agitated at room temperature for 1 h. 5-Aminobenzotriazole was added and the mixture agitated at room temperature for 16 h. The resin was filtered and washed according to the standard protocol 8.2.3 to obtain the title compound as colourless beads. In this case, double coupling was carried out to ensure complete coupling.

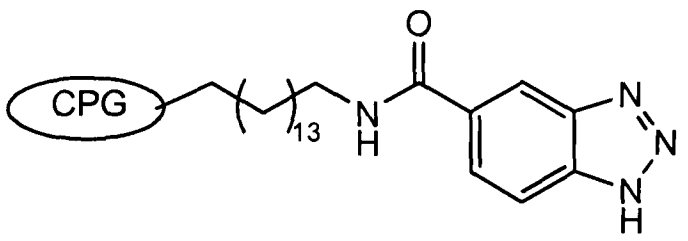
υ_{max} (KBr disc)/ cm^{-1}, 1658 (CO, amide I), 1580 (CO, amide II).

8.6.7 1H-Benzotriazole-5-carboxylic acid-amino-propyl CPG



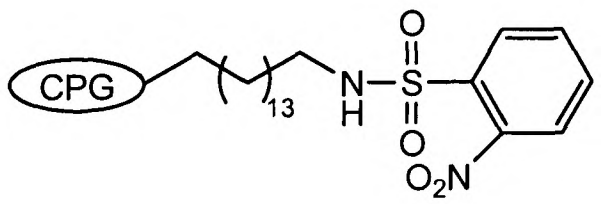
To a solution of benzotriazole-5-carboxylic acid **207** (0.19 g, 1.2 mmol, 5eq.) in dimethylformamide (2 mL) was added, HBTU (0.45 g, 1.2 mmol, 4.9eq.) and HOBt (0.16 g, 1.2 mmol, 5eq.), followed by diisopropylethylamine (0.43 mL, 0.31 g, 2.4 mmol, 10eq.) with stirring. The coupling mixture was immediately added to the amino-propyl CPG (1011 Å pore size, 29.9 m^2 g^{-1}, surface area 125-177 μm particle size) (80.3 μmol g^{-1}, 3.00 g, 0.24 mmol, 1eq.). The reaction mixture was agitated on a blood rotator for 16 h to obtain brown beads (73.0 μmol g^{-1}, L_{max} = 79.3 μmol g^{-1}, 92% loading by elemental analysis). Kaiser 8.2.5.1 test confirmed coupling.
υ_{max} (KBr disc)/ cm^{-1}, 1632 (CO, amide I).

8.6.8 1H-Benzotriazole-5-carboxylic acid-long chain amino-propyl CPG 299



To a solution of benzotriazole-5-carboxylic acid **207** (0.25 g, 1.5 mmol, 5eq.) in dimethylformamide (5 mL) was added, HBTU (0.57 g, 1.5 mmol, 4.9eq.) and HOBt (0.21 g, 1.5 mmol, 5eq.), followed by diisopropylethylamine (0.55 mL, 0.40 g, 3.1 mmol, 10eq.) with stirring. The coupling mixture was immediately added to the long chain amino-propyl CPG **298** (1088 Å pore size, 34.4 m^2 g^{-1} surface area, 125-177 μm particle size) (77.5 μmol g^{-1}, 4.0 g, 0.31 mmol, 1eq.). The reaction mixture was agitated on a blood rotator for 16h to obtain brown beads (48.0 μmol g^{-1}, 63% loading by elemental analysis). Kaiser test (Section 8.2.5.1) confirmed coupling.
υ_{max} (KBr disc)/ cm^{-1}; 1634 (CO, amide I).

8.6.9 *Attempted synthesis of 2-nitrobenzenesulfonamide coupled long chain amino-propyl CPG 304*

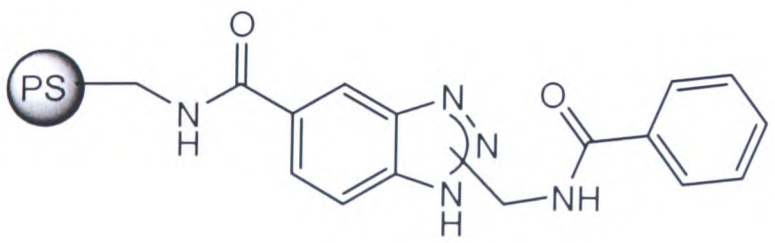


To a suspension of **298** (1088 Å pore size, 34.4 m^2 g^{-1} surface area, 125-177 μm particle size) (77.5 μmol g^{-1}, 0.45 g, 35 μmol, 1eq.) in THF/DCM (3 mL) and DMAP (26 μL, 0.21 mmol, 6eq.), was added 2-nitrobenzene sulfonyl chloride (31 mg, 0.14 mmol, 4eq.). The resulting suspension was agitated on a blood rotator at room temperature for 1.5 h. After this time, the Kaiser test (8.2.5.1) revealed incomplete coupling. The reaction mixture was filtered, washed with DCM and fresh reagents added. The reaction was repeated a further 4 times, however the Kaiser test showed a positive result each time. Sulfur analysis confirmed the failure of the 2-nitrobenzene sulfonyl coupling.

8.6.10 *General procedure for preparation of aminal linkers*[106]

Desired aldehyde (10eq.), phenylacetamide (10eq.) and *p*-toluene sulfonic acid (0.5eq.) were dissolved in toluene. Benzotriazole resin (1eq.) was added and the reaction mixture was heated under reflux for 48 h using a Dean-Stark apparatus for azeotropic removal of water. The resin was filtered and washed according to the standard protocol 8.2.3, unless otherwise stated.

8.6.11 *1-(Benzoylamino-methyl)-1H-benzotriazole-carboxyamino-polystyrene 232*



General procedure 8.6.10 was followed using formaldehyde (37% in H_2O/MeOH) (0.66 mL, 0.73 g, 24 mmol), benzamide (2.94 g, 24.3 mmol), *p*-toluene

sulfonic acid (0.23 g, 1.2 mmol) and **218** (assume 1.23 mmol g^{-1}, 1.98 g, 2.43 mmol) in anhydrous toluene (50 mL) to obtain the desired product as brown beads. 0.52 mmol g^{-1} by acid cleavage (Section 8.2.7.1), followed by quantitation of the benzamide released by reverse phase HPLC (Section 8.2.9), L_{max} = 0.90 mmol g^{-1}, 58% loading.

υ_{max} (KBr disc)/ cm^{-1} 3057, 3023 (CH, aryl), 2919, 2850 (sat. CH), 1645 (CO, amide I), 1600 (CH, aryl), 1580 (CO, amide II). δ_H (MAS-NMR; $CDCl_3$; 600 MHz) 5.43 (CH_2), 8.30 (CH_{bt}), 7.80 (CH_{bt}), 7.35 (CH_{arom}), 7.24 (CH_{arom}).

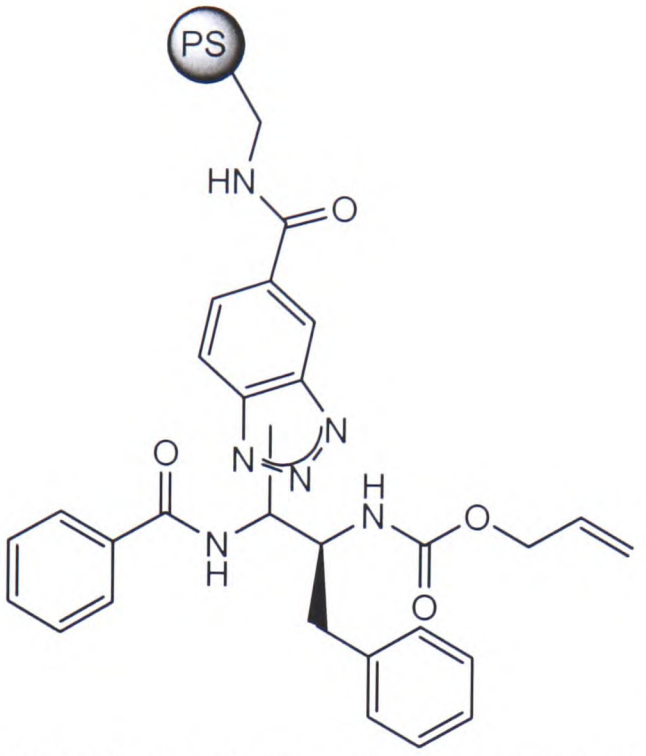
8.6.11.1 Optimisation of acid cleavage

The cleavage of **232** was carried out in plastic isolute tubes using either trifluoroacetic acid/dichloromethane/water (9:10:1) or 95% trifluoroacetic acid/water cleavage mixtures. For each cleavage mixture, 11 aliquots of resin **232** (~13 mg) were treated with the cleavage mixture (0.5 mL). After 30 min, the first sample was filtered, washed with acetonitrile/water (1:1) (5 mL), concentrated and dissolved in acetonitrile/water (1:1) (5 mL), then the amount of benzamide released was analysed by reverse phase HPLC. This was repeated every 15 min over 4.75 h. The amount of benzamide released against time was plotted for both cleavage methods. Controls contained all reagents except the acid and were allowed to run until the end of the experiment before being analysed.

HPLC conditions: Using equipment according to section 8.2.9 with an isocratic method eluting with H_2O/CH_3CN/TFA (7:3:0.01). Benzamide standards were prepared and a concordancy test performed.

Analyte	**R_t(min)**
Benzamide	3.9

8.6.12 *1-[1-({2S}-Allyloxycarbonylamino-1-benzoylamino-3-phenyl-propyl)-1H-benzotriazole]-carboxyamino-polystyrene 233*



General procedure 8.6.10 was followed using **171** (1.80 g, 7.80 mmol, 5eq.), benzamide (0.94 g, 7.8 mmol, 5eq.), *p*-toluene sulfonic acid (70 mg 0.39 mmol) and **218** (assume 1.23 mmol g^{-1}, 1.27 g, 1.56 mmol, 1eq.) in anhydrous toluene (50 mL). The resin was filtered and washed with copious amounts of DMF and DCM (>300 mL).

υ_{max} (KBr disc)/ cm^{-1}, 3023 (CH aryl), 2923 (sat. CH), 1714 (CO, urethane), 1645 (CO, amide I), 1539 (CO, amide II); δ_H (MAS-NMR; $CDCl_3$; 600 MHz) 4.30-4.67 (CH_2CH= and $CHCH_2Ph$), 4.90-5.24 (CH_2=), 7.86 (CH_{bt}).

8.6.12.1 Determination of enantiomeric purity of cleaved aldehyde

A large sample of resin **233** (238 mg) was treated with TFA/DCM/H_2O (9:10:1) (0.5 mL) for 4 h. The resin was filtered, washed with acetonitrile/water (1:1) and concentrated. The residue was dissolved in ethylacetate and sodium borohydride (5.2 mg) was added. The solution was stirred at room temperature for 30 min. After this time, the reaction mixture was concentrated and dissolved in *iso*-propyl alcohol

before being analysed by normal phase chiral HPLC and compared to a sample of racemic allyloxycarbonyl-phenylalaninol **175**.

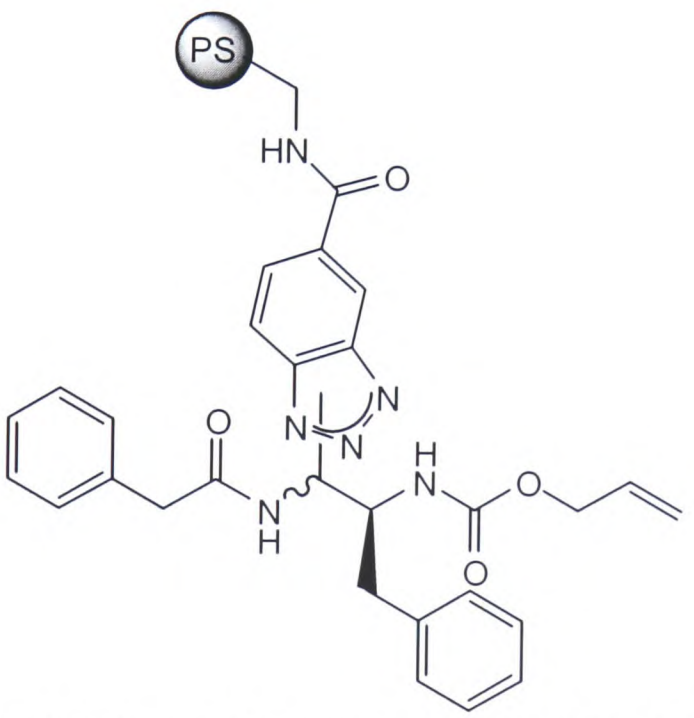
Chiral HPLC confirmed that the alcohol **174** was almost chirally pure (97% e.e.).

Chiral HPLC conditions: Using equipment according to section 8.2.9 with an isocratic system eluting with hexane/IPA (98:2) at 10 °C.

Specificity of HPLC method:

Analyte	R_t (min)
(*S*)-**174**	30.5
(*R*)- **174**	36.8

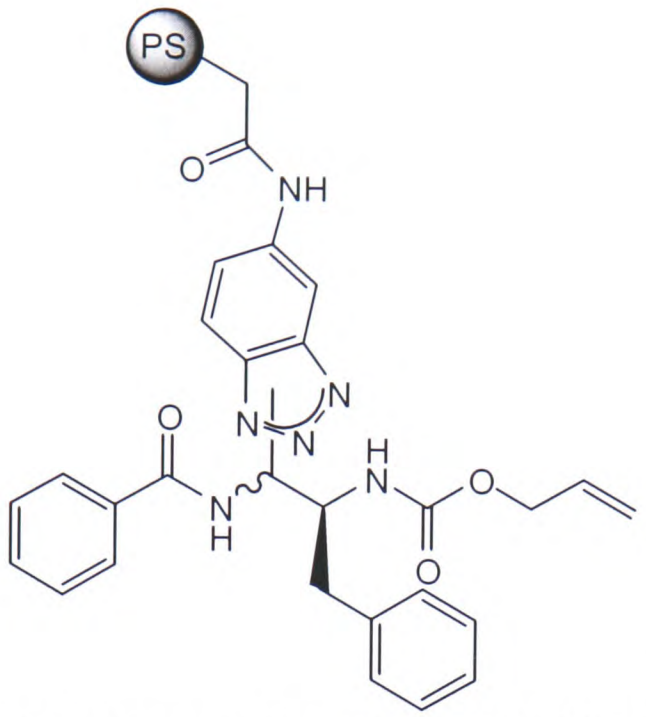
8.6.13 1-[1-({2S}-Allyloxycarbonylamino-1-phenylacetylamino-3-phenyl-propyl)-1H-benzotriazole]-carboxyamino-polystyrene 234



General procedure 8.6.10 was followed using (*S*)-**171** (0.32 g, 1.4 mmol, 6eq.), phenylacetamide (0.19 g, 1.4 mmol, 6eq.), *p*-toluene sulfonic acid (0.02 g, 0.1 mmol, 0.5eq.) and **218** (assume 1.23 mmol g^{-1}, 0.19 g, 0.2 mmol, 1eq.) in anhydrous toluene (50 mL). The resin was filtered and washed with copious amounts of DMF and DCM (>300 mL).

υ_{max} (KBr disc)/ cm^{-1}, 1703 (CO, urethane), 1652 (CO, amide I), 1600 (CH, aryl), 1521 (CO, amide II); δ_C ($CDCl_3$; 63 MHz) 145.8 (broad, C_{bt}).

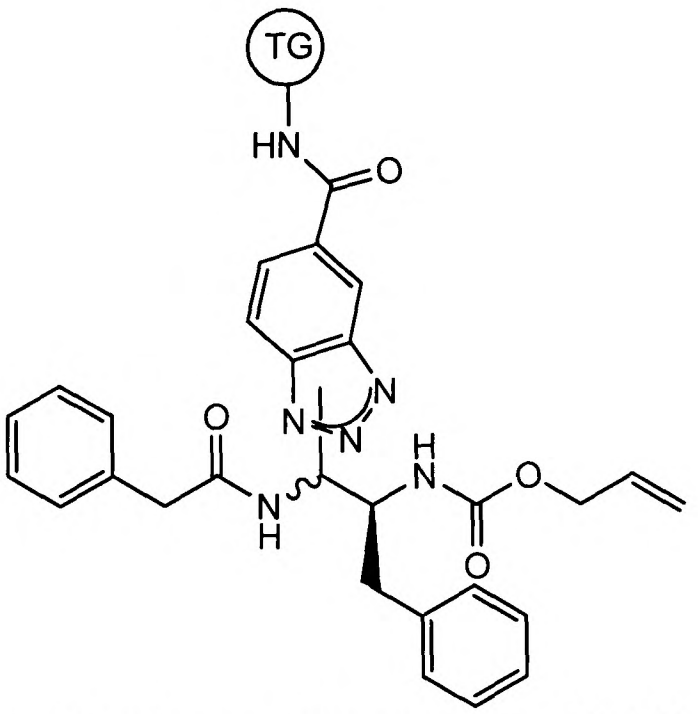
8.6.14 *1-[1-({2S}-Allyloxycarbonylamino-1-phenylacetylamino-3-phenyl-propyl)-1H-benzotriazole]-amino-carboxypolystyrene 255*



General procedure 8.6.10 was followed using (*S*)-**171** (2.5 g, 11 mmol, 10eq.), benzamide (1.3 g, 11 mmol, 10eq.), *p*-toluene sulfonic acid (0.09 g, 0.5 mmol) and **254** (assume 1.24 mmol g^{-1}, 0.86 g, 1.1 mmol, 1eq.) in anhydrous toluene (40 mL). The resin was filtered and washed with copious amounts of DMF and DCM (>300 mL).

υ_{max} (KBr disc)/ cm^{-1} 3081, 3059, 3024 (CH aryl), 2922 (sat. CH), 1715 (CO, urethane), 1650 (CO, amide I), 1604 (CH aryl), 1538 (CO, amide II).

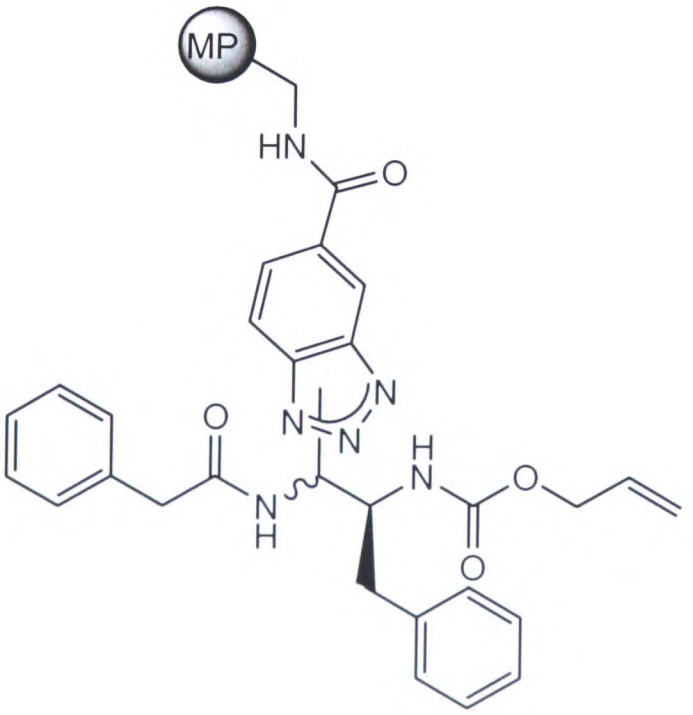
8.6.15 1-[1-({2S}-Allyloxycarbonylamino-1-phenylacetylamino-3-phenyl-propyl)-1H-benzotriazole]-carboxyamino-TentaGel 238



General procedure 8.6.10 was followed using (*S*)-**171** (0.60 g, 2.6 mmol), phenylacetamide (0.35 g, 2.6 mmol), *p*-toluene sulfonic acid (0.02 g, 0.10 mmol) and **217** (assume 0.45 mmol g^{-1}, 0.57 g, 0.30 mmol). The resin was filtered and washed with copious amounts of DMF and DCM (>300 mL).

υ_{max} (KBr disc)/ cm^{-1}, 3025 (=CH stretch), 2920 (sat. CH), 1717 (CO, urethane), 1635 (CO, amide I), 1541 (CO, amide II).

8.6.16 1-[1-({2S}-Allyloxycarbonylamino-1-phenylacetylamino-3-phenyl-propyl)-1H-benzotriazole]-carboxyamino-macroporous polystyrene 237



General procedure 8.6.10 was followed using (*S*)-**171** (0.35 g, 1.5 mmol, 10eq.), phenylacetamide (0.20 g, 1.5 mmol, 10eq.), *p*-toluene sulfonic acid (0.01 g, 0.07 mmol, 0.5eq.) and **219** (assume 1.19 mmol g^{-1}, 0.12 g, 0.15 mmol, 1eq.) in anhydrous toluene (13 mL). The resin was filtered and washed with copious amounts of DMF and DCM (>300 mL).

υ_{max} (KBr disc)/ cm^{-1} 3020 (CH, aryl), 2926 (sat. CH), 1718 (masked CO, urethane), 1664 (CO, amide I), 1600 (CH, aryl), 1509 (CO, amide II).

*8.6.16.1 Enzymatic studies on TentaGel and macroporous polystyrene-bound benzotriazole substrates **238** and **237**.*

Hydrolysis experiments were carried out in plastic isolute tubes fitted with a plastic cap and tip. To aliquots of the resin-bound benzotriazole substrates **237** and **238** (~10 mg) was added penicillin acylase (~0.8 mg) in potassium phosphate buffer pH 7.5 (10 mM) (1 mL). The suspensions were rotated for 16 h at room temperature on a 360° blood rotator. The suspensions were filtered, washed with ~10 mL acetonitrile/water (1:1) and concentrated. The resulting residues were dissolved in acetonitrile/water (1:1) (2 mL) for analysis by reverse-phase HPLC. The control experiment contained all reagents except the enzyme.

Acid cleavage: To an aliquot of the resin-bound benzotriazole substrate **237** and **238** (~500 mg) was added TFA/DCM/H_2O (9:10:1) (1 mL). The suspensions were rotated for 16 h at room temperature on a 360° blood rotator. The suspensions were filtered, washed with ~10 mL acetonitrile/water (1:1) and concentrated. The resulting residues were dissolved in acetonitrile/water (1:1) (2 mL) for analysis by reverse-phase HPLC. The control experiment contained all reagents except acid.

HPLC conditions: Using equipment according to Section 8.2.9 with an isocratic method eluting with H_2O/CH_3CN/TFA (7:3:0.01).

Specificity of HPLC method

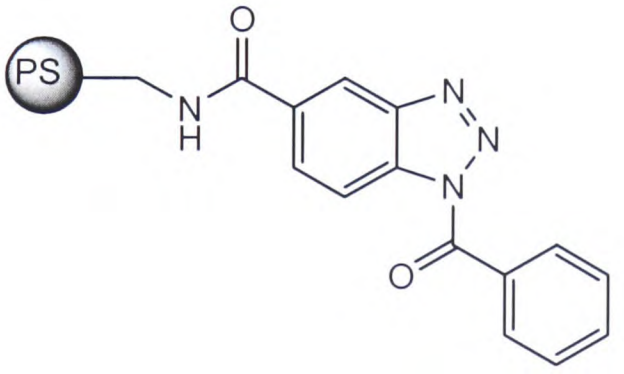
Analyte	R_t (min)
171	9.6
Phenylacetic acid	6.6
Phenylacetamide	4.2

Table 10: *Specificity of HPLC method for substrates.*

8.6.17 General procedure for preparation of N-acyl benzotriazole resins[134]

To a suspension of the desired benzotriazole-resin (1eq.) in anhydrous dichloromethane was added triethylamine (10eq.), followed by the relevant acid chloride (10eq.), dropwise at 0 °C under argon. The resulting mixture was stirred at room temperature for 20 h. The resin was filtered and washed according to the standard protocol 8.2.3.

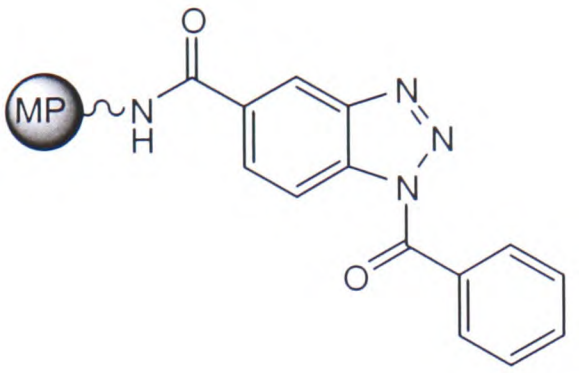
8.6.18 1-(1-Benzoyl-1H-benzotriazole)-carboxyamino-polystyrene 281



General procedure 8.6.17 was carried out using **218** (assume 1.23 mmol g^{-1}, 0.52 g, 0.64 mmol), benzoyl chloride (0.74 mL, 0.90g, 6.4 mmol) and triethylamine (0.9 mL, 0.65g, 6.4 mmol) in anhydrous dichloromethane (25 mL).

υ_{max} (KBr disc)/ cm^{-1}, 3058, 3024 (CH, aryl), 2921 (sat. CH), 1717 (CO, *N*-acyl), 1652 (CO, amide I), 1599 (CH, aryl), 1521 (CO, amide II); δ_H (MAS-NMR; $CDCl_3$; 600 MHz) 8.70 (CH_{bt}), 8.51 (CH_{bt}), 8.35 (CH_{bt}), 8.11 (CH_{arom}), 7.59 (CH_{arom}), 7.46 (CH_{arom}).

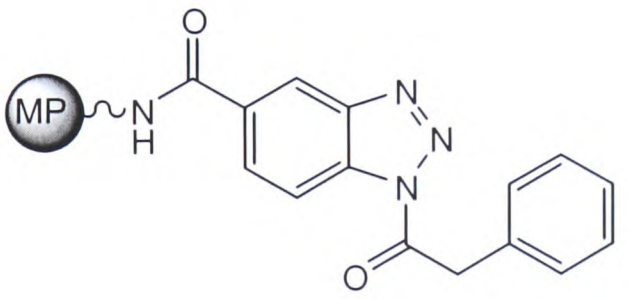
8.6.19 1-(1-Benzoyl-1H-benzotriazole)-carboxyamino-macroporous polystyrene 282



General procedure 8.6.17 was carried out using **219** (assume 1.19 mmol g^{-1}, 0.50 g, 0.59 mmol), benzoyl chloride (0.69 mL, 0.83 g, 5.9 mmol) and triethylamine (0.84 mL, 0.60 g, 5.9 mmol) in anhydrous dichloromethane (25 mL).

υ_{max} (KBr disc)/ cm^{-1}, 3020 (CH, aryl), 2924 (sat. CH), 1717 (CO, *N*-acyl), 1645 (CO, amide I), 1603 (CH, aryl), 1509 (CO, amide II).

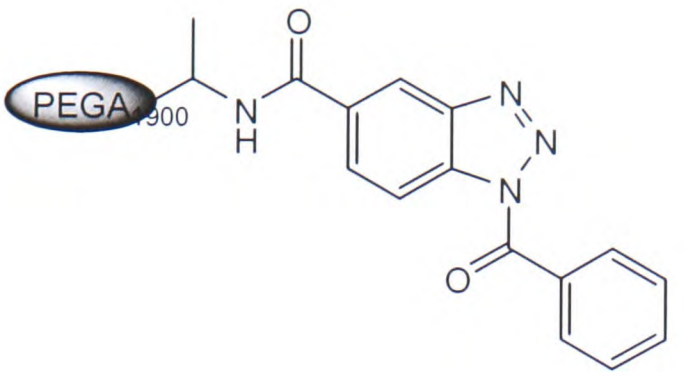
8.6.20 1-(1-Phenylacetyl-1H-benzotriazole)-carboxyamino-macroporous polystyrene 283



General procedure 8.6.17 was carried out using **219** (assume 1.19 mmol g^{-1}, 0.47 g, 0.56 mmol), phenylacetyl chloride (0.74 mL, 0.86 g, 5.6 mmol) and triethylamine (0.78 mL, 0.57 g, 5.6 mmol) in anhydrous dichloromethane (25 mL).

υ_{max} (KBr disc)/ cm^{-1}, 3022 (CH, aryl), 2924 (sat. CH), 1718 (CO, *N*-acyl), 1644 (CO, amide I), 1604 (CH, aryl), 1510 (CO, amide II).

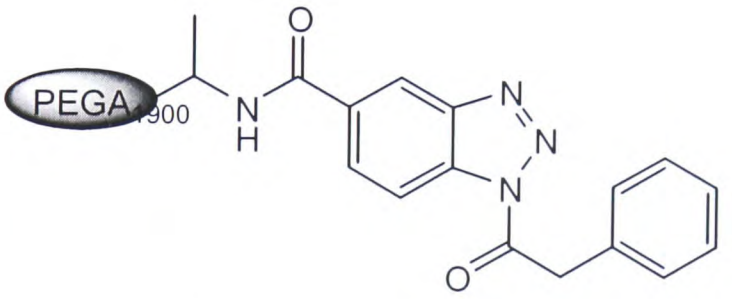
8.6.21 1-(1-Benzoyl-1H-benzotriazole)-carboxyamino-PEGA$_{1900}$ 284



General procedure 8.6.17 was carried out using **220** (assume 0.2 mmol g^{-1}, 0.5 g, 0.1 mmol), benzoyl chloride (0.14 g, 0.12 mL, 1.0 mmol) and triethylamine (0.14 mL, 0.10 g, 1.0 mmol) in anhydrous dichloromethane (25 mL).

υ_{max} (KBr disc)/ cm^{-1} 2986 (sat. CH), 1661 (CO, *N*-acyl), 1653 (CO, amide I), 1557 (CH, aryl), 1507 (CO, amide II); δ_H (MAS-NMR; $CDCl_3$; 600 MHz) 8.80 (C*H*$_{bt}$), 8.23 (C*H*$_{bt}$ x 2), 8.41 (C*H*$_{bt}$), 8.23 (*C*H$_{arom}$ x 2), 7.72 (*C*H$_{arom}$), 7.60 (*C*H$_{arom}$).

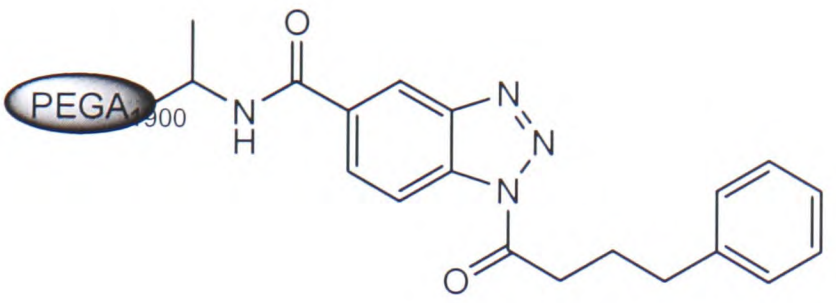
8.6.22 1-(1-Phenylacetyl-1H-benzotriazole)-carboxyamino-PEGA$_{1900}$ 285



General procedure 8.6.17 was carried out using **220** (assume 0.2 mmol g^{-1}, 0.5 g, 0.1 mmol), phenylacetyl chloride (0.15 g, 0.13 mL, 1.0 mmol) and triethylamine (0.14 mL, 0.10 g, 1.0 mmol) in anhydrous dichloromethane (25 mL).

υ_{max} (KBr disc)/ cm^{-1} 2986 (sat. CH), 1690 (CO, *N*-acyl), 1650 (CO, amide I), 1601 (CH, aryl), 1507 (CO, amide II); δ_H (MAS-NMR; $CDCl_3$; 600 MHz) 8.60-7.20 (combination of C*H*$_{bt}$ and C*H*$_{arom}$), 4.61 (C*H*$_2$Ph).

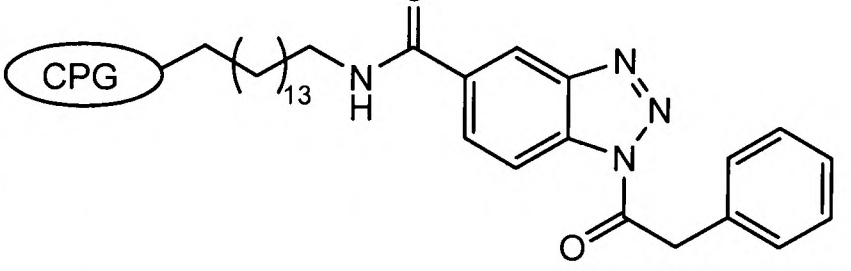
8.6.23 *1-[1-(4-Phenylacetyl)-1H-benzotriazole]-carboxyamino-PEGA$_{1900}$ 286*



General procedure 8.6.17 was carried out using **220** (assume 0.2 mmol g^{-1}, 1.14 g, 0.230 mmol), 4-phenylbutyryl chloride (0.42 g, 2.3 mmol) and triethylamine (0.32 mL, 0.23 g, 2.3 mmol) in anhydrous dichloromethane (50 mL).

υ_{max} (KBr disc)/ cm^{-1} 2985 (sat. CH), 1689 (CO, *N*-acyl), 1651 (CO, amide I), 1600 (CH, aryl), 1505 (CO, amide II); δ_H (MAS-NMR; $CDCl_3$; 600 MHz) 2.35 (CH_2), 8.55-7.10 (combination of CH_{bt} and CH_{arom}).

8.6.24 *1-(1-Phenylacetyl-1H-benzotriazole)-carboxyamino-long chain amino-propyl CPG 300*



General procedure 8.6.17 was carried out using **299** (assume 77.5 μmol g^{-1}, 4.0 g, 0.31 mmol), phenylacetyl chloride (0.48 g, 0.41 mL, 3.1 mmol) and triethylamine (0.43 mL, 0.31 g, 3.1 mmol) in anhydrous dichloromethane (100 mL).

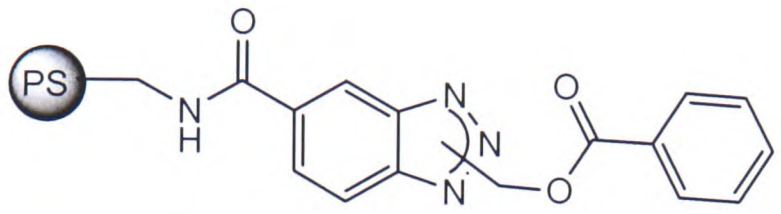
υ_{max} (KBr disc)/ cm^{-1} 2985 (sat. CH), 1690 (CO, *N*-acyl), 1640 (CO, amide I), 1603 (CH, aryl), 1500 (CO, amide II);

8.6.25 *General procedure for the preparation of hemiaminal linkers*

To a suspension of *N*-acyl benzotriazole resin (1eq.) and desired aldehyde (8-10eq.) in acetonitrile was added triethylamine (3eq.) with vigorous shaking. The

resulting mixture was agitated on a blood rotator at room temperature for 20 h. The resin was washed according to the standard protocol.

8.6.26 *1-[1-(Benzyloxycarbonyl-methyl)-1H-benzotriazole]-carboxyamino-polystyrene 287*



General procedure 8.6.25 was followed with **284** (assume 1.23 mmol g^{-1}, 0.98 g, 1.2 mmol), formaldehyde (37% solution in H_2O/MeOH) (0.26 mL, 0.29 g, 9.6 mmol), triethylamine (0.51 mL, 0.36 g, 3.6 mmol) in acetonitrile (5 mL) to afford the title compound as pale brown beads (701 μmol g^{-1} loading from basic cleavage (1 M NaOH/Dioxane 3:2), L_{max} = 914 μmol g^{-1}, 77%).

υ_{max} (KBr disc)/ cm^{-1} 3058,3023 (CH, aryl), 2919, 2850 (sat. CH), 1731 (CO, ester), 1645 (CO, amide I), 1600 (CH, aryl), 1521 (CO, amide II). δ_H (MAS-NMR; $CDCl_3$; 600 MHz) 6.55 (CH_2), 7.31 (CH_{arom}), 7.46 (CH_{arom}), 7.95, 7.88 (CH_{bt} x 2), 8.41 (CH_{bt}).

8.6.27 *Evaluation of chemical cleavage methods for polymer bound benzotriazole hemiaminal 287*

Cleavage experiments were performed in 2 mL plastic isolute tubes. The procedures for each base cleavage are detailed below:

8.6.27.1 Methanolic ammonia:

An aliquot of dry resin **287** (12 mg) was swollen in DMF. A solution of ammonia in methanol (7 mmol l^{-1}, 0.3 mL) was added at 0 °C. The tube was sealed and allowed to warm to room temperature before being agitated for 18 h on a 360° blood rotator. The resin was filtered, washed with methanol (5 mL) and the mother liquor evaporated to dryness.

8.6.27.2 *Hydrazine hydrate:*

An aliquot of dry resin **287** (15 mg) was suspended in DMF. A solution of 5% hydrazine hydrate in DMF (0.3 mL) was added. The suspension was agitated on a 360° blood rotator at room temperature for 1 h. The resin was filtered, washed with DMF (5 mL) and the mother liquor evaporated to dryness.

8.6.27.3 *DIPEA:*

An aliquot of dry resin **287** (12 mg) was suspended in DMF. A solution of DIPEA/MeOH/DMF (1:5:5) (0.6 mL) was added. The suspension was agitated on a 360° blood rotator at room temperature for 16 h. The resin was filtered, washed with MeOH/DMF (5 mL) and the mother liquor evaporated to dryness.

8.6.27.4 *Sodium methoxide:*

An aliquot of dry resin **287** (13 mg) was suspended in DMF. A solution of 1 M sodium methoxide in H_2O/dioxane (1:3) (0.3 mL) was added. The suspension was agitated on a 360° blood rotator at room temperature for 1 h. The resin was filtered, washed with MeOH and the mother liquor evaporated to dryness.

8.6.27.5 *Sodium hydroxide:*

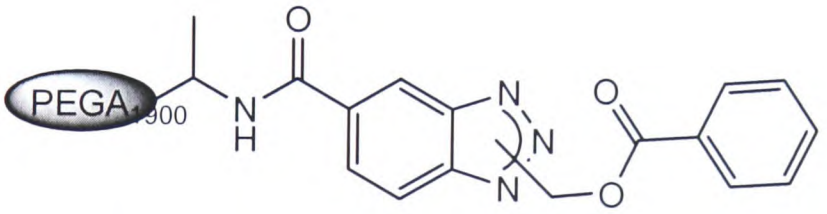
A solution of 1 M sodium hydroxide/dioxane (1:3) was cooled to 0 °C in an ice/water bath. An aliquot of dry resin **287** (11 mg) was added and the resulting suspension was agitated on a 360° blood rotator at room temperature for 1 h. The resin was filtered into a flask containing 1 M HCl (0.2 mL), washed with water and the mother liquor evaporated to dryness.

HPLC procedures and conditions: The residues were diluted with acetonitrile/water (50:50) (5 mL) and analysed using reverse phase HPLC. Using equipment according to Section 8.2.8 with isocratic elutions according to Table 11.

Cleavage mixture	**Analyte**	**Isocratic method**	**R_t (min)**
Methanolic ammonia	Benzamide	H_2O/CH_3CN / TFA (7:3:0.01)	4.7
Hydrazine hydrate	Benzoic hydrazide	H_2O/CH_3CN / TFA (8:2:0.01)	4.1
Diisopropylethylamine/MeOH	Methyl benzoate	H_2O/CH_3CN / TFA (1:1:0.01)	7.2
Sodium methoxide	Methyl benzoate	H_2O/CH_3CN / TFA (1:1:0.01)	7.2
Sodium hydroxide	Benzoic acid	H_2O/CH_3CN / TFA (7:3:0.01)	7.9

Table 11: *RP-HPLC methods for base cleavage procedures.*

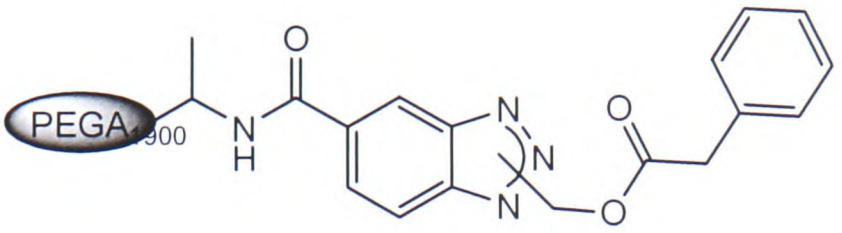
8.6.28 1-[1-(Benzyloxycarbonyl-methyl)-1H-benzotriazole]-carboxyamino-PEGA$_{1900}$ 288



General procedure 8.6.25 was followed with **284** (assume 0.2 mmol g^{-1}, 0.50 g, 0.10 mmol), formaldehyde (37% solution in H_2O/MeOH) (0.02 mL, 0.02 g, 0.8 mmol), triethylamine (0.04 mL, 0.03 g, 0.3 mmol) in acetonitrile (5 mL) to afford the title compound as pale brown beads (65 μmol g^{-1} loading from basic cleavage (1 M NaOH/Dioxane 3:2), L_{max}= 189 μmol g^{-1}, 34% loading).

υ_{max} (KBr disc)/ cm^{-1} 2922, 2869 (sat. CH), 1731 (CO, ester), 1662 (CO, amide I), 1604 (CH, aryl), 1557 (CO, amide II). δ_H (MAS-NMR; $CDCl_3$; 600 MHz) 7.05 (CH_2), 7.65-8.45 (combination of CH_{bt} and CH_{arom}).

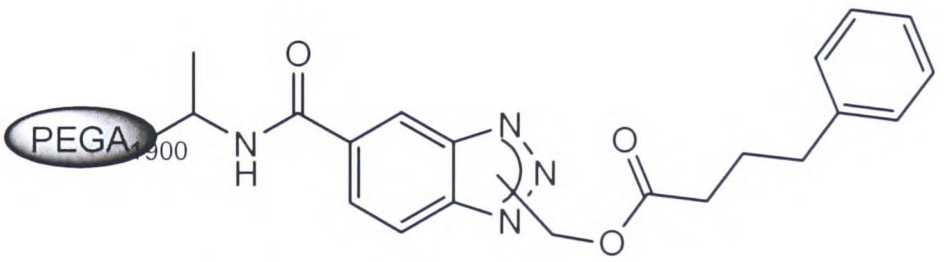
8.6.29 *1-[1-(Phenylacetyloxycarbonyl-methyl)-1H-benzotriazole]-carboxyamino-PEGA$_{1900}$ 289*



General procedure 8.6.25 was followed with **285** (assume 0.2 mmol g^{-1}, 0.4 g, 0.08 mmol), formaldehyde (37% solution in H_2O/MeOH) (0.02 mL, 0.02 g, 0.6 mmol), triethylamine (0.03 mL, 0.02 g, 0.3 mmol) in acetonitrile (5 mL) to afford the title compound as pale brown beads.

υ_{max} (KBr disc)/ cm^{-1} 2920, 2870 (sat. CH), 1745 (CO, ester), 1660 (CO, amide I), 1600 (CH, aryl), 1555 (CO, amide II). δ_H (MAS-NMR; $CDCl_3$; 600 MHz) 6.75 (CH_2O), 7.55-8.24 (combination of CH_{bt} and CH_{arom}).

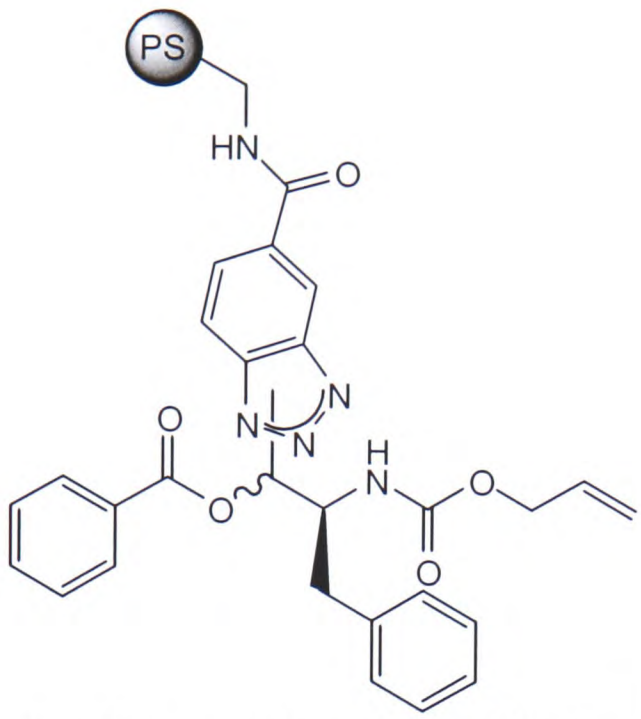
8.6.30 *1[1-({4-Phenylbutyryloxycarbonyl}-methyl)-1H-benzotriazole]-carboxyamino-PEGA$_{1900}$ 290*



General procedure 8.6.25 was followed with **286** (assume 0.2 mmol g^{-1}, 0.78 g, 0.15 mmol), formaldehyde (37% solution in H_2O/MeOH) (0.03 mL, 0.04 g, 1 mmol), triethylamine (0.06 mL, 0.05 g, 0.5 mmol) in acetonitrile (5 mL) to afford the title compound as pale brown beads.

υ_{max} (KBr disc)/ cm^{-1} 2922, 2870 (sat. CH), 1740 (CO, ester), 1660 (CO, amide I), 1600 (CH, aryl), 1554 (CO, amide II). δ_H (MAS-NMR; $CDCl_3$; 600 MHz) 2.51 (CH_2), 6.63 (CH_2O), 7.12-8.14 (combination of CH_{bt} and CH_{arom}).

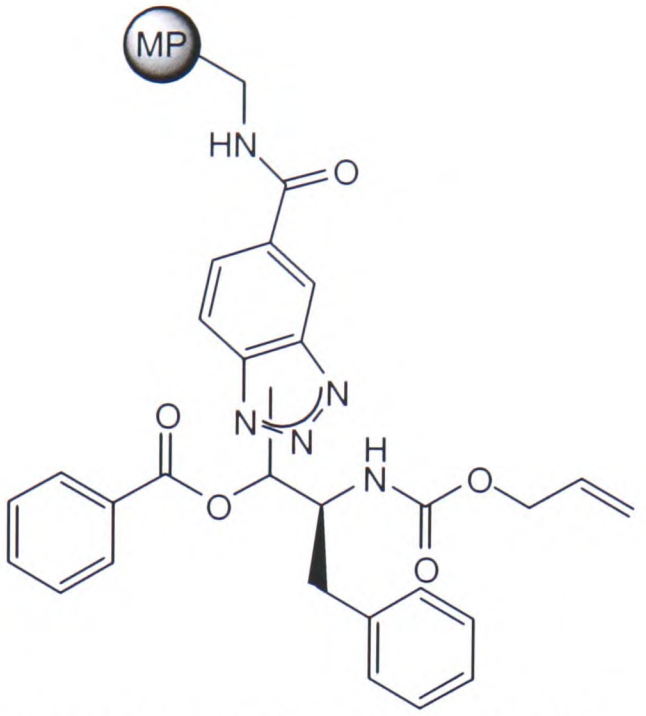
8.6.31 1-[1-({2S}-Allyloxycarbonylamino-1-benzyloxycarbonyl-3-phenyl-propyl)-1H-benzotriazole]-carboxyamino-polystyrene 293



General procedure 8.6.25 was followed with **281** (assume 1.23 mmol g^{-1}, 1.79 g, 2.20 mmol), **171** (2.56 g, 11.0 mmol), triethylamine (0.92 mL, 0.67 g, 6.6 mmol) in acetonitrile (5 mL) to afford the title compound as brown beads.

υ_{max} (KBr disc)/ cm^{-1} 3059, 3023 (CH, aryl), 2921, 2850 (sat. CH), 1731 (CO, ester and urethane), 1644 (CO, amide I), 1539 (CO, amide II). δ_H (MAS-NMR; $CDCl_3$; 600 MHz) 2.88 (CH_2Ph), 4.32 (CHCH_2Ph), 4.54 (CH_2CH=), 5.06 (CH_2=), 5.75 (CH=), 7.92-8.51 (CH_{bt} x 3), 7.32-7.61 (CH_{arom} x 10).

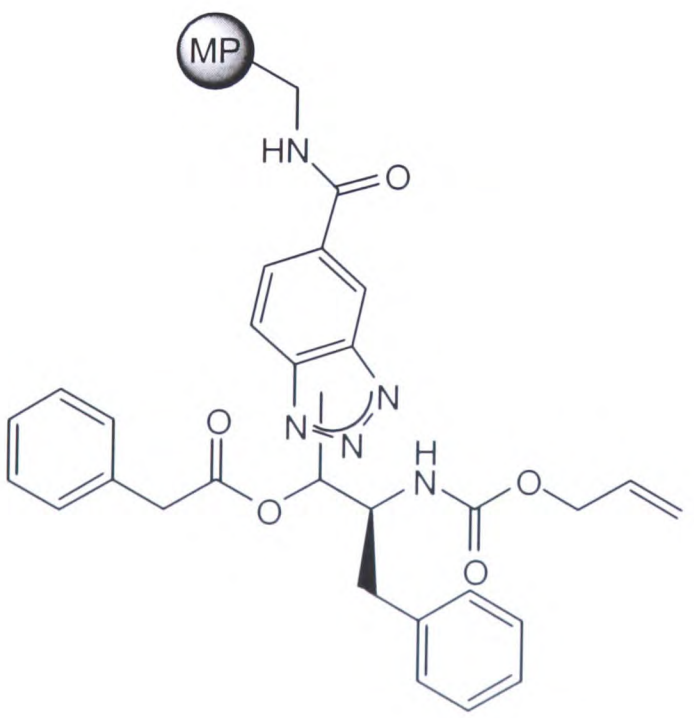
8.6.32 *1-[1-({2S}-Allyloxycarbonylamino-1-benzyloxycarbonyl-3-phenyl-propyl)-1H-benzotriazole]-carboxyamino-macroporous polystyrene*



General procedure 8.6.25 was followed with **282** (assume 1.19 mmol g^{-1}, 0.53 g, 0.63 mmol), (*S*)-**171** (1.47 g, 6.31 mmol), triethylamine (0.27 mL, 0.19 g, 1.9 mmol) in acetonitrile (10 mL) to obtain the title compound as pale brown beads.

υ_{max} (KBr disc)/ cm^{-1} 3021 (CH, aryl), 2925 (sat. CH), 1718 (CO, ester and urethane), 1644 (CO, amide I), 1603 (CH, aryl), 1510 (CO, amide II).

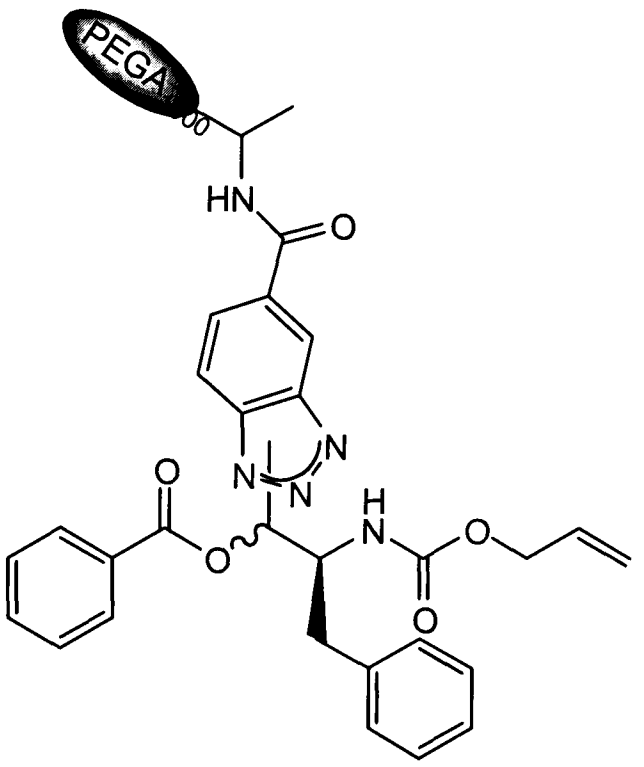
8.6.33 *1-[1-({2S}-Allyloxycarbonylamino-1-phenylacetyloxycarbonyl-3-phenyl-propyl)-1H-benzotriazole]-carboxyamino-macroporous polystyrene*



General procedure 8.6.25 was followed with **283** (assume 1.19 mmol g^{-1}, 0.47 g, 0.56 mmol), (*S*)-**171** (1.29 g, 5.56 mmol), triethylamine (0.23 mL, 0.16 g, 1.7 mmol) in acetonitrile (10 mL) to obtain the title compound as pale brown beads.

υ_{max} (KBr disc)/ cm^{-1} 3022 (CH, aryl), 2924 (sat. CH), 1718 (CO, ester and urethane), 1645 (CO, amide I), 1604 (CH, aryl), 1510 (CO, amide II).

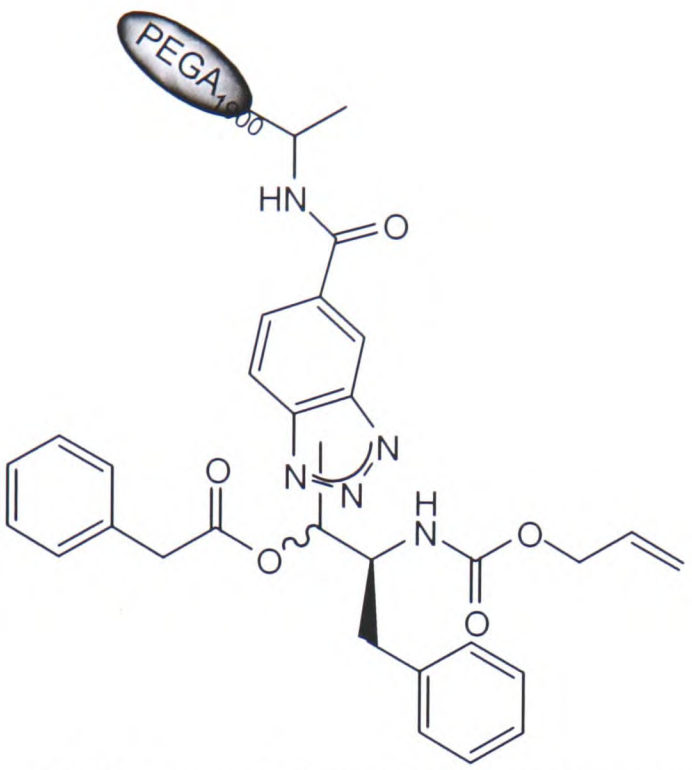
8.6.34 1-[1-({2S}-Allyloxycarbonylamino-1-benzyloxycarbonyl-3-phenyl-propyl)-1H-benzotriazole]-carboxyamino-PEGA$_{1900}$ 295



General procedure 8.6.25 was followed with **284** (assume 0.2 mmol g^{-1}, 0.093 g, 19 μmol), (*S*)-**171** (0.035 g, 0.15 mmol), triethylamine (8.0 μL, 6.0 mg, 56 μmol) in acetonitrile (5 mL) to obtain the title compound as a pale brown gel.

υ_{max} (KBr disc)/ cm^{-1} 3016 (CH, aryl), 1725 (CO, ester and urethane), 1650 (CO, amide I), 1520 (CO, amide II). δ_H (MAS-NMR; $CDCl_3$; 600 MHz) 5.56, 5.37, 5.34, 5.22, 5.20, 5.07, 4.92, 4.24 (various peaks corresponding to C*H*$_2$=, C*H*=, C*H*N and C*H*$_2$CH=), 6.89 (C*H*O), 7.02, 7.16, 7.17, 7.25, 7.32, 7.47, 7.46 (C*H*$_{arom}$), 7.90 (C*H*$_{bt}$ x 2), 8.35 (C*H*$_{bt}$).

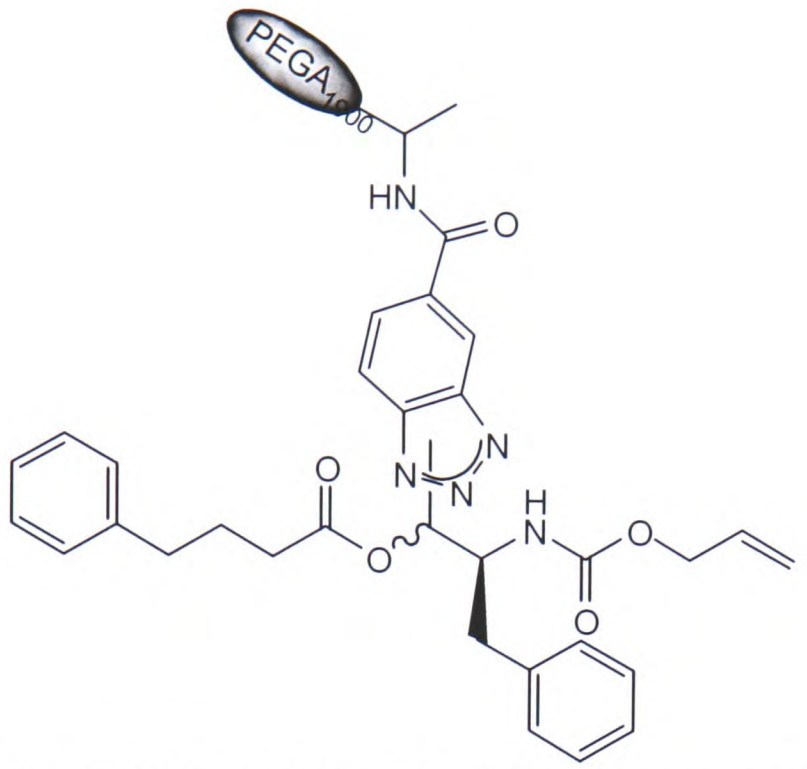
8.6.35 *1-[1-({2S}-Allyloxycarbonylamino-1-phenylacetyloxycarbonyl-3-phenyl-propyl)-1H-benzotriazole]-carboxyamino-PEGA$_{1900}$ 296*



General procedure 8.6.25 was followed with **285** (assume 0.2 mmol g^{-1}, 1.1 g, 0.23 mmol), (*S*)-**171** (0.27 g, 1.1 mmol), triethylamine (90 μL, 69 mg, 0.69 mmol) in acetonitrile (25 mL) to obtain the title compound as a pale brown gel.

υ_{max} (KBr disc)/ cm^{-1} 2919 (sat. CH), 1700 (CO ester and urethane), 1646 (broad CO, amide I), 1540 (CO, amide II). δ_H (MAS-NMR; $CDCl_3$; 600 MHz) 4.24 (C*H*$_2$CH=), 4.73 (C*H*N), 5.15 (C*H*$_2$=), 5.75 (C*H*=), 6.92 (C*H*O), 7.02-7.45 (C*H*$_{arom}$ x 10), 7.50-8.32 (C*H*$_{bt}$ x 3).

8.6.36 *1-[1-({2S}-Allyloxycarbonylamino-1-{4-phenylbutyryloxycarbonyl}-3-phenyl-propyl)-1H-benzotriazole]-carboxyamino-PEGA$_{1900}$ 297*



General procedure 8.6.25 was followed with **286** (assume 0.2 mmol g^{-1}, 1.14 g, 230 μmol), (*S*)-**171** (270 mg, 1.14 mmol), triethylamine (90 μL, 69 mg, 0.68 mmol) in acetonitrile (25 mL) to obtain the title compound as a pale brown gel.

υ_{max} (KBr disc)/ cm^{-1} 3021 (CH, aryl), 1720 (CO, ester and urethane), 1635 (CO, amide I), 1505 (CO, amide II); δ_H (MAS-NMR; $CDCl_3$; 600 MHz) 4.00 (C*H*$_2$CH=), 5.30 (C*H*$_2$=), 5.83 (C*H*=), 7.40-8.06 (C*H*$_{bt}$).

8.6.37 *Enzymatic studies on PEGA$_{1900}$-bound benzotriazole substrates*

8.6.37.1 *Screening of enzymes for hydrolysis of PEGA$_{1900}$-bound benzotriazole substrates **288-290** and **295-297**.*

Hydrolysis experiments were carried out in plastic isolute tubes fitted with a plastic cap and tip. To an aliquot of the PEGA$_{1900}$-bound benzotriazole substrate, **288-290** and **295-297** (~500 mg = 50 mg dry) was added the enzyme (~0.8 mg) in potassium phosphate buffer pH 7.5 (10 mM)/acetonitrile (9:1) (1 mL). The suspensions were rotated for 16 h at room temperature on a 360° blood rotator. The suspensions were filtered, washed with ~10 mL acetonitrile/water (1:1) and concentrated. The resulting residues were dissolved in acetonitrile/water (1:1) (2

mL) for analysis by reverse-phase HPLC. The control experiment contained all reagents except the enzyme. Control and enzyme hydrolyses were repeated with all substrates.

Base cleavage: To an aliquot of the $PEGA_{1900}$-bound benzotriazole substrate, **288-290** and **295-297** (~500 mg = 50 mg dry) was added 7 M ammonia in methanol (1 mL). The suspensions were rotated for 16 h at room temperature on a 360° blood rotator. The suspensions were filtered, washed with ~10 mL acetonitrile/water (1:1) and concentrated. The resulting residues were dissolved in acetonitrile/water (1:1) (2 mL) for analysis by reverse-phase HPLC. The control experiment contained all reagents except ammonia. Control and base hydrolyses were repeated with all substrates.

HPLC conditions: Using equipment according to Section 8.2.9 with an isocratic method eluting with H_2O/CH_3CN/TFA (7:3:0.01).

Specificity of HPLC method

Analyte	**R_t (min)**
171	9.6
Benzoic acid	6.7
Benzamide	3.9
Phenylacetic acid	6.6
Phenylacetamide	4.2
4-Phenylbutyric acid	15.1
4-Phenylbutyramide	5.3

Table 12: *Specificity of HPLC method for substrates **288-290** and **295-297**.*.

8.6.37.2 *Determination of enantiomeric excess of **171** for enzymatic cleavage of **296**.*

To an aliquot of **296** (2.70 g = 270 mg dry) was added a solution of the enzyme (0.8 mg in potassium phosphate buffer pH 7.5 (10 mM)/acetonitrile (9:1)) (1 mL). The suspensions were rotated for 16 h at room temperature on a 360° blood rotator. The suspensions were filtered, washed with ~10 mL acetonitrile/water (1:1) and

concentrated. The resulting residues were reduced according to Section 8.3.18 and the resulting alcohol **174** was dissolved in *iso*-propanol/hexane (1:1) (2 mL) for analysis by chiral normal phase HPLC. The control experiment contained all reagents except the enzyme.

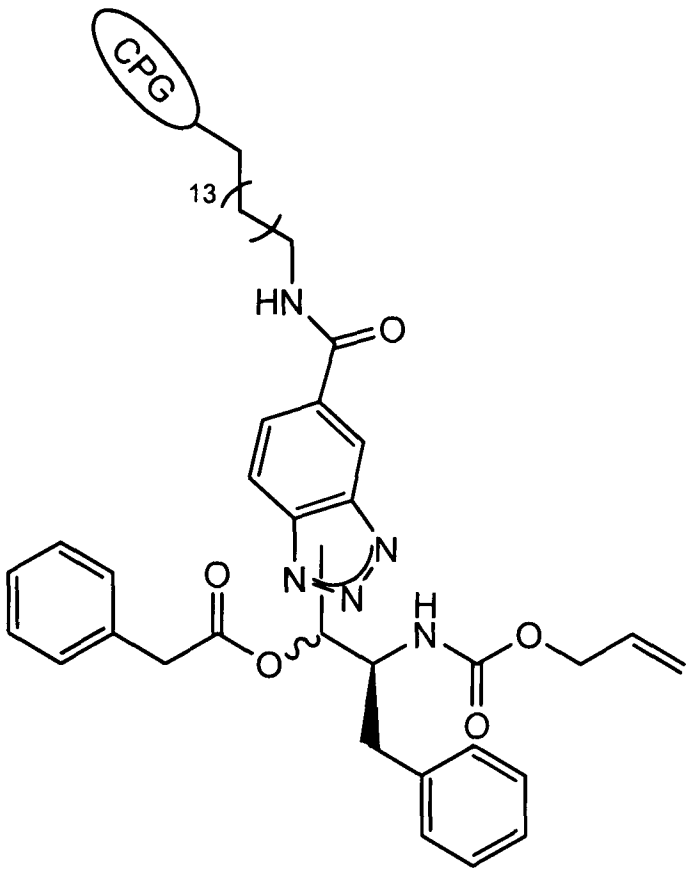
Normal phase chiral HPLC conditions: Using equipment according to Section 8.2.9 with a Chiracel OD-H column with a gradient elution at 10 °C. The gradient elution method is shown below.

Time (min)	Hexane	IPA
0	99	1
5	99	1
40	95	5
42	99	1
45	99	1

Specificity of chiral HPLC method:

Analyte	**R_t (min)**
(*S*)-**174**	35.3
(*R*)-**174**	38.9

8.6.38 *1-[1-({2S}-Allyloxycarbonylamino-1-phenylacetyloxycarbonyl-3-phenyl-propyl)-1H-benzotriazole]-carboxyamino-long chain amino propyl CPG 301*

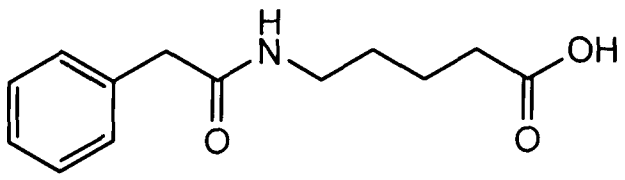


General procedure 8.6.25 was followed with **300** (assume 77.5 mmol g^{-1}, 3.8 g, 0.29 mmol), (*S*)-**171** (0.34 g, 1.5 mmol), triethylamine (0.12 mL, 90 mg, 0.88 mmol) in acetonitrile (100 mL) to yield the desired compound as pale brown beads (40 μmol g^{-1}, 53% as calculated from elemental analysis).

υ_{max} (KBr disc)/ cm^{-1} 3019 (CH, aryl), 1725 (CO, urethane and ester), 1641 (CO, amide I).

8.7 Preparation of safety catch linker

8.7.1 *5-Phenylacetylamino-pentanoic acid 323*

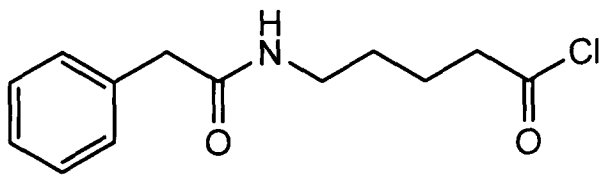


To a solution of 2-aminovaleric acid (2.0 g, 17 mmol) in 4 M sodium hydroxide (3.1 mL) was added in eight portions, phenylacyl chloride (2.6 g, 2.2 mL, 17 mmol) in 4 M sodium hydroxide (3.1 mL) at 0 °C, with a few minutes between each

addition. The reaction mixture was stirred for 15 min, allowed to stand at room temperature for 2 h. The reaction mixture was extracted with diethyl ether and the aqueous layer was acidified with concentrated HCl (1 mL). The precipitate that formed was removed by filtration, washed with hexane and dried *in vacuo* to give the desired product as a white solid (2.0 g, 50%).

Mpt 65.5-72.0 °C; R_f 0.18 [methanol/chloroform (2:1)]; υ_{max} (nujol)/ cm^{-1} 3295 (OH, H-bonded), 1689 (CO, carboxylic acid), 1630 (CO, amide I), 1541 (CO, amide II); δ_H ($CDCl_3$; 250 MHz) 1.46-1.64 (4H, overlapping tt, *J*=6.6, 6.6 Hz, $NHCH_2CH_2$ and CH_2CH_2CO), 2.34 (2H, t, *J*=6.9 Hz, CH_2CO) 3.23 (2H, dt, *J*=6.6, 6.1 Hz, CH_2NH), 3.61 (2H, CH_2Ph), 7.25-7.38 (5H, m, Ph); δ_C ($CDCl_3$; 63 MHz) 21.5 (CH_2), 28.6 (CH_2), 33.2 (CH_2), 39.0 (CH_2), 43.5 (CH_2), 127.3 (CH_{arom}), 128.9 (CH_{arom} x 2), 129.4 (CH_{arom} x 2), 133.3 (*C*), 171.6 ($C{=}O_{amide}$), 178.8 ($C{=}O_{acid}$); *m/z* (ES-MS; -ve), 234 (100%, $M\text{-}H^-$); FAB-MS, Found: MH^+ 236.1289, $C_{13}H_{18}NO_3$ requires MH^+ 236.1286.

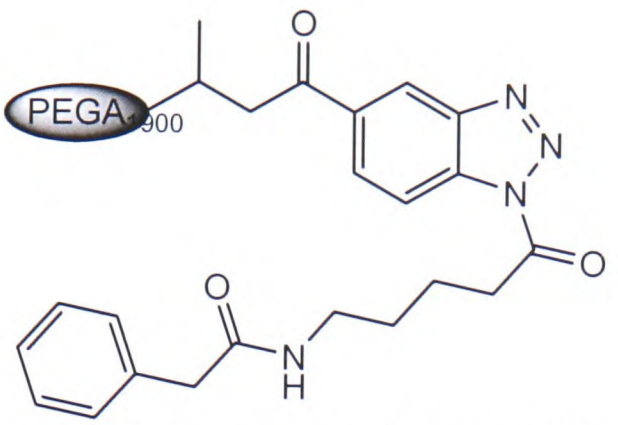
8.7.2 5-Phenylacetylamino-pentanoyl chloride 324



To a solution of **323** (5.0 g, 21 mmol) in anhydrous dichloromethane (50 mL) was added oxalyl chloride (3.2 g, 2.2 mL, 25 mmol), followed by catalytic dimethylformamide (1 drop). The reaction was allowed to stir at room temperature for 1 h, evaporated to remove any excess oxalyl chloride, to provide the crude product as a moisture sensitive colourless oil (1.6 g, 30%).

υ_{max} (DCM film)/ cm^{-1} 1815 (CO, acid chloride), 1638 (CO, amide I).

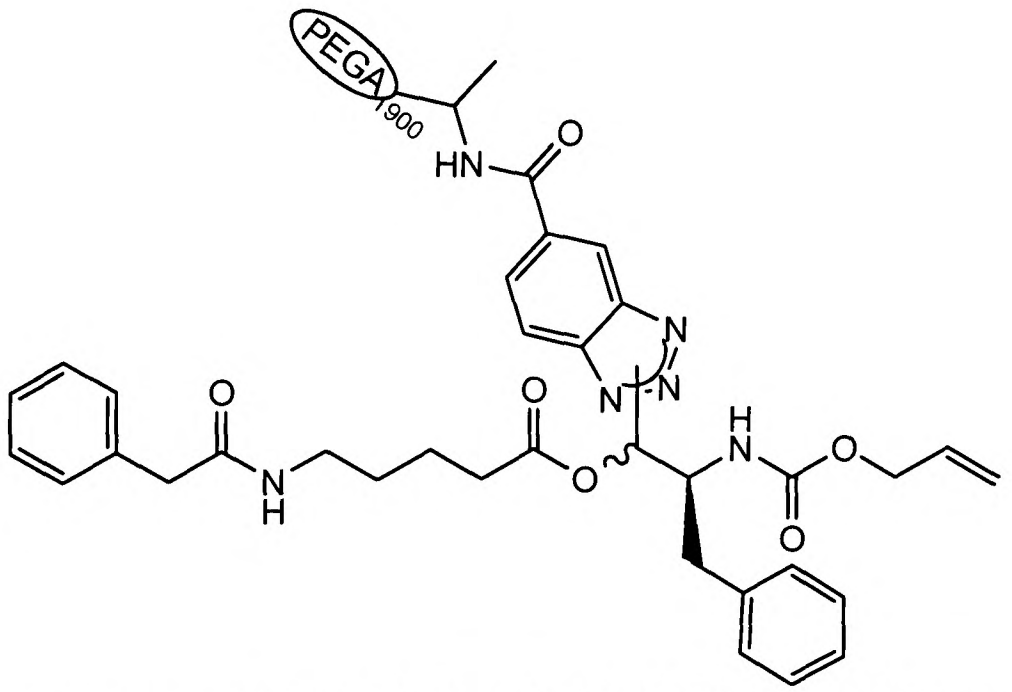
8.7.3 *1-[1-(5-Phenylacetylamino-pentanoyl)-1H-benzotriazole]-carboxyamino-PEGA$_{1900}$ 325*



General procedure 8.6.16.1 was carried out using **220** (assume 0.2 mmol g^{-1}, 0.66 g, 0.13 mmol), **324** (0.34 g, 1.3 mmol) and triethylamine (0.18 mL, 0.13 g, 1.3 mmol) in anhydrous dichloromethane (5 mL).

υ_{max} (KBr disc)/ cm^{-1} 2985 (sat. CH), 1720 (CO, *N*-acyl), 1650 (CO, amide I), 1602 (CH, aryl), 1515 (CO, amide II).

8.7.4 *1-[1-({2S}-Allyloxycarbonylamino-1-{5-phenylacetylamino-pentanyloxycarbonyl}-3-phenyl-propyl)-1H-benzotriazole]-carboxyamino-PEGA$_{1900}$ 326*



General procedure 8.6.25 was followed with **325** (assume 0.2 mmol g^{-1}, 0.39 g, 0.078 mmol), (*S*)-**171** (0.091 g, 0.39 mmol), triethylamine (0.03 mL, 0.02 g, 0.2 mmol) in acetonitrile (5 mL) to obtain the title compound as a pale brown gel.

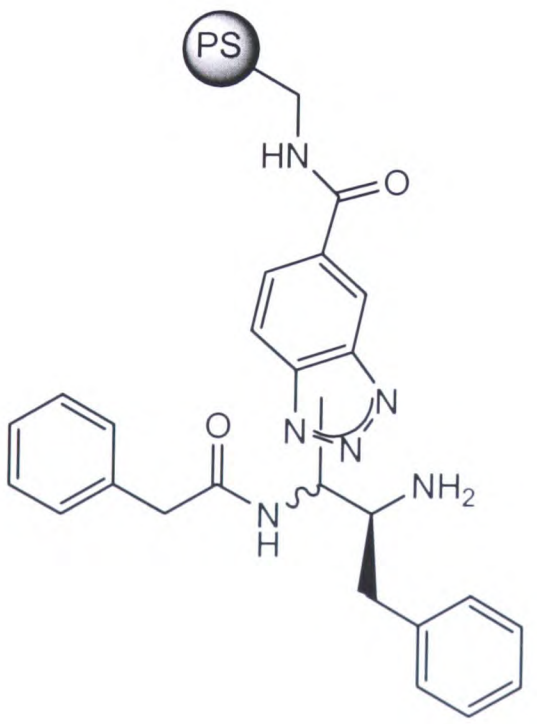
υ_{max} (KBr disc)/ cm^{-1} 3020 (CH, aryl), 2923 (sat. CH), 1740 (CO, ester and urethane), 1646 (CO, amide I), 1600 (CH, aryl), 1515 (CO, amide II). δ_H (MAS-NMR; $CDCl_3$; 600 MHz) 1.90 (C*H*$_2$), 4.24 (C*H*$_2$CH=), 6.10 (C*H*=), 6.85 (C*H*O), 7.02-7.45 (C*H*$_{arom}$ x 10), 7.50-8.32 (C*H*$_{bt}$ x 3).

8.8 Solid phase synthesis of peptide aldehydes

8.8.1 General procedure for solid phase Alloc removal[95]

Borane-dimethylamine complex (6eq.) was added to Alloc-protected resin (1eq.) in anhydrous dichloromethane under agitation, followed a few minutes later, by tetra*kis*(triphenylphosphine)palladium (0) (10 mol%). After 10 min, at room temperature, the suspension was drained and washed with dichloromethane. The deprotection procedure was repeated until the Kaiser test 8.2.5.1 gave a blue solution. The resin was washed successively with dichloromethane/trifluoroacetic acid (1drop), dichloromethane/diisopropylethylamine (1 drop) and dichloromethane.

8.8.2 1-[1-({2S}-Amino-1-phenylacetylamino-3-phenyl-propyl)-1H-benzotriazole]-carboxyamino-polystyrene 246



General procedure 8.8.1 was followed using **234** (assume 1.23 mmol g^{-1}, 0.16 g, 0.20 mmol), tetra*kis*(triphenylphosphine)palladium (0) (0.02 g, 0.02 mmol) and borane-dimethyl amine complex (70 mg, 1.2 mmol) in dichloromethane (3 mL). The deprotection procedure was repeated to give the title compound as brown beads.

υ_{max} (KBr disc)/ cm^{-1} 3059 (CH, aryl), 1650 (CO, amide I).

8.8.3 General procedure for peptide coupling

Fmoc-amino acid (5eq.) was dissolved in dimethylformamide and diisopropylethylamine (8eq.) was added with stirring. The pre-mixed 0.45 M HBTU/HOBt reagent (8.8.4) (5eq.) was then added with stirring and the activated amino acid solution was transferred to the reaction vessel containing the resin (1eq.). The suspension was agitated on a blood rotator for ~4 h at room temperature. The resin was filtered and washed with dimethylformamide and dichloromethane. Coupling was complete when a straw brown colour was observed under Kaiser conditions 8.2.5.1. The coupling procedure was repeated, if necessary.

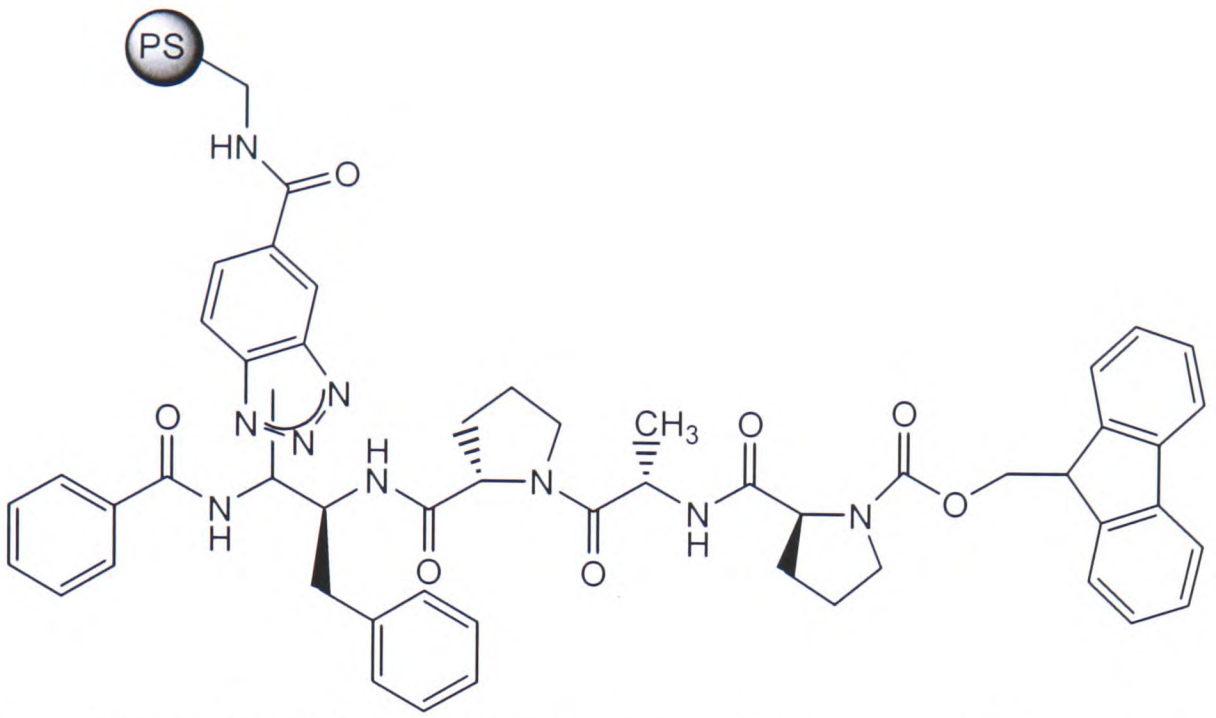
8.8.4 Preparation of 0.45M HBTU/HOBt solution

HBTU (9.4 g) was added to a solution of HOBt (3.4 g) in dimethylformamide (50 mL). The mixture was stored at 4 °C for up to 2 months.

8.8.5 General procedure for Fmoc deprotection

A sample of washed resin was treated with 20% piperidine in dimethylformamide, over a period of 30 min. The reaction mixture was drained and washed with dimethylformamide and dichloromethane. Complete deprotection was achieved when a blue colour was observed using the Kaiser test 8.2.5.1. The deprotection procedure was repeated, if necessary.

8.8.6 *Attempted sythesis of tetrapeptide bound to 1-[1-({2S}-amino-1-benzylamino-3-phenyl-propyl)-1H-benzotriazole]-carboxyamino-polystyrene 249*

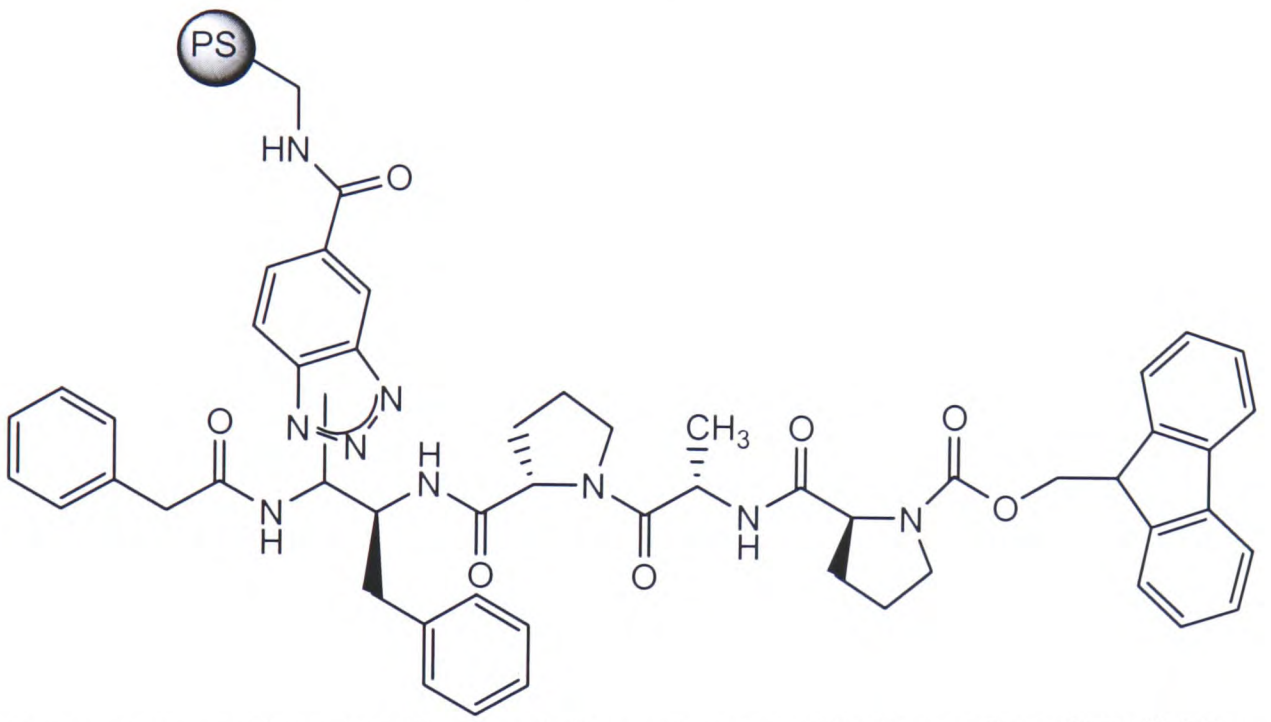


General procedure 8.8.1 was followed using **233** (assume 1.23 mmol g^{-1}, 0.50 g, 0.62 mmol), tetra*kis*(triphenylphosphine)palladium (0) (0.07 g, 0.06 mmol) and borane-dimethyl amine complex (0.22 g, 3.7 mmol) in dichloromethane (10 mL). The deprotection procedure was repeated to give the title compound as brown beads. General procedure 8.8.3 was followed using (*S*)-Fmoc-Pro-OH (1.04 g, 3.07 mmol), free amine resin (0.50 g, 0.62 mmol), 0.45 M HBTU/HOBt (6.8 mL, 3.1 mmol) and diisopropylethylamine (0.86 mL, 4.9 mmol) to obtain the Fmoc-Pro-coupled peptide (0.38 mmol g^{-1}, L_{max} = 0.65 mmol g^{-1}, 58% loading by Fmoc analysis 8.2.6.1). The Fmoc group was removed using general procedure 8.8.5 and (*S*)-Fmoc-Ala-OH (1.01 g, 3.07 mmol) was coupled by repeating procedure 8.8.3 using similar quantities of reagents. The final amino acid was coupled by removal of the Fmoc group (Section 8.8.5) and addition of (*S*)-Fmoc-Pro-OH (0.54 g, 1.6 mmol) as described before 8.8.3. The polymer-bound tetra-peptide was washed exhaustively with dimethylformamide and dichloromethane and dried *in vacuo.* 0.16 mmol g^{-1} loading by Fmoc analysis 8.2.6.1, L_{max} = 0.58 mmol g^{-1}, 27% loading.

υ_{max} (KBr disc)/ cm^{-1} 3080, 3058, 3023 (CH, aryl), 2921, 2851 (sat. CH), 1714 (CO urethane). 1651 (CO, amide I), 1600 (CH, aryl),1515 (CO, amide II). δ_H (MAS-NMR; $CDCl_3$; 600 MHz) 7.63 (C*H*$_{bt}$), 7.74 (C*H*$_{bt}$), 5.78 (NC*H*N), 4.20-4.60 (broad unresolved, CHC*H*CH_2, OCH_2, various CH from proline and alanine)

An aliquot of resin **249** (3.5 mg) was cleaved under acidic conditions 8.2.7.1. The residue obtained was dissolved in acetonitrile/water (1:1) (5 mL) and electrospray mass spectrometry as described in sections 8.2.9 and 8.2.8. No peak corresponding to the peptide aldehyde was evident in the electrospray mass spectrometry.

8.8.7 Tetrapeptide bound to 1-[1-({2S}-amino-1-phenylacetylamino-3-phenyl-propyl)-1H-benzotriazole]-carboxyamino-polystyrene 250

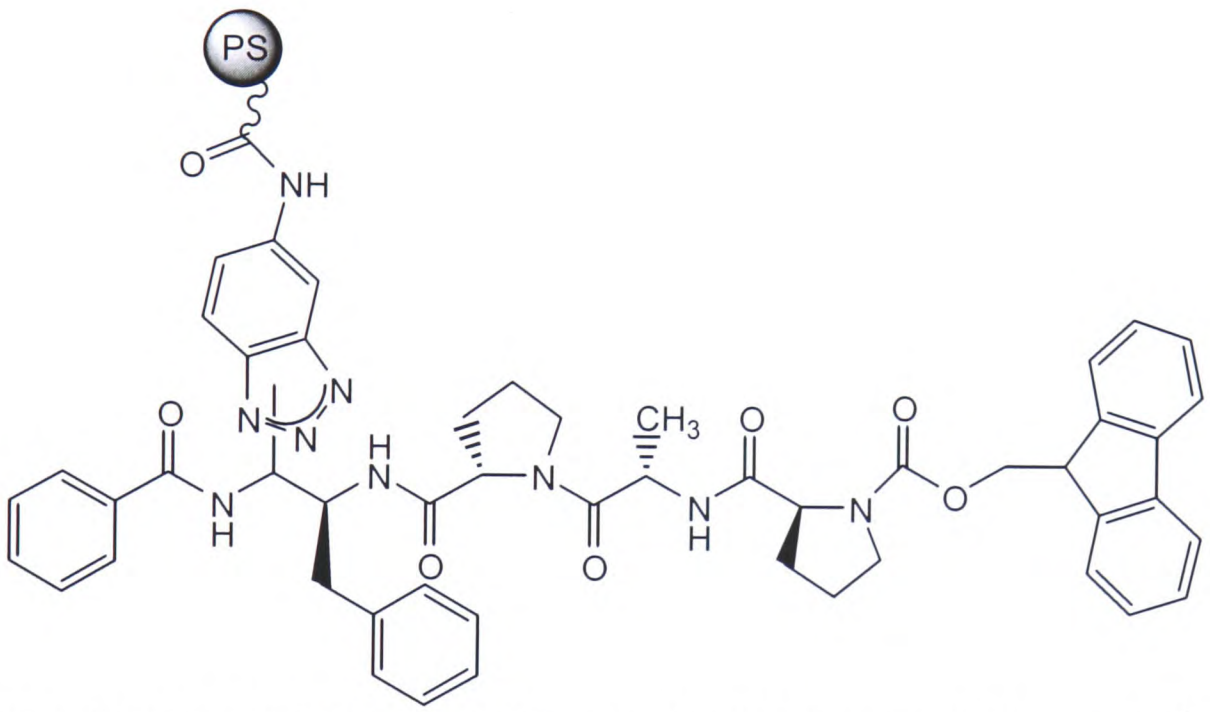


General procedure 8.8.3 was followed using (*S*)-Fmoc-Pro-OH (0.33 g, 0.98 mmol), **246** (0.16 g, 0.19 mmol), 0.45 M HBTU/HOBt (2.2 mL, 0.98 mmol) and diisopropylethylamine (0.27 mL, 1.57 mmol) to obtain the Fmoc-Pro-OH coupled peptide. The Fmoc group was removed using general procedure 8.8.5 and (*S*)-Fmoc-Ala-OH (0.32 g, 0.98 mmol) was coupled by repeating procedure 8.8.3. The final amino acid was coupled by removal of the Fmoc group 8.8.5 and addition of (*S*)-Fmoc-Pro-OH (0.33 g, 0.98 mmol) as before 8.8.3. The polymer-bound tetra-peptide was washed exhaustively with dimethylformamide and dichloromethane and dried *in vacuo.* (0.28 mmol g^{-1} by Fmoc analysis 8.2.6.1, L_{max}= 0.58 mmol g^{-1}, 48% loading). δ_H (MAS-NMR; $CDCl_3$; 600 MHz) 7.61 (C*H*$_{bt}$), 7.73 (C*H*$_{bt}$), 5.97 (NC*H*N), 4.20-4.60 (broad unresolved, CHC*H*CH_2, OC*H*$_2$, various C*H* from proline and alanine.

An aliquot of resin **250** (5 mg) was cleaved under acidic conditions 8.2.7.1. The residue obtained was dissolved in acetonitrile/water (1:1) and analysed by LC-MS as described in section 8.2.8.

m/z (LC-MS; +ve) 637 (40%, MH^+, R_t = 26.0 min), 675 (5%, MNa^+). A second acidic cleavage showed no presence of further cleavage indicating complete cleavage.

8.8.8 *Tetrapeptide bound to 1-[1-({2S}-amino-1-benzylamino-3-phenyl-propyl)-1H-benzotriazole]-aminocarboxy-polystyrene 256*



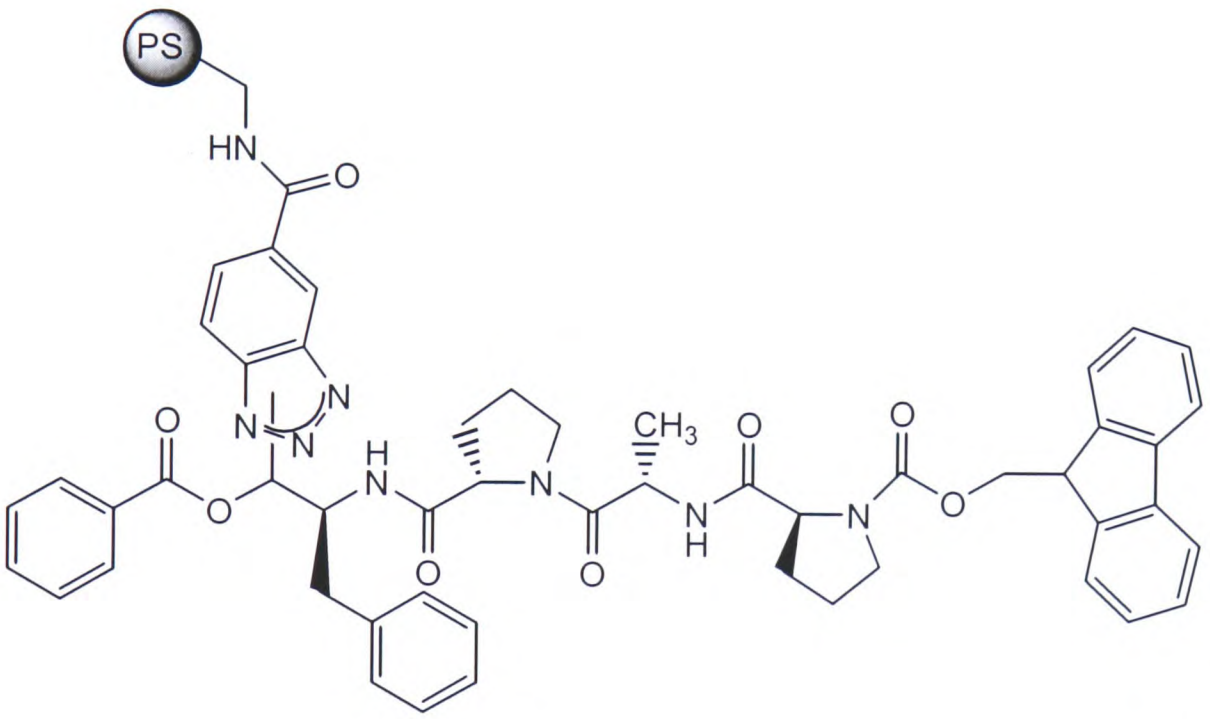
General procedure 8.8.1 was followed using **255** (assume 1.24 mmol g^{-1}, 0.53 g, 0.65 mmol), tetra*kis*(triphenylphosphine)palladium (0) (0.07 g, 0.06 mmol) and borane-dimethyl amine complex (0.23 g, 3.9 mmol) in dichloromethane (5 mL). The deprotection procedure was repeated another twice to give the title compound as brown beads. General procedure 8.8.3 was followed using (*S*)-Fmoc-Pro-OH (0.83 g, 2.4 mmol), free amine resin (0.5 g, 0.6 mmol), 0.45 M HBTU/HOBt (5.4 mL, 2.4 mmol) and diisopropylethylamine (0.75 mL, 4.3 mmol) to obtain the Fmoc-Pro-coupled peptide. The Fmoc group was removed using general procedure 8.8.5 and (*S*)-Fmoc-Ala-OH (0.81 g, 2.4 mmol) was coupled by repeating procedure 8.8.3 using similar quantities of reagents. The final amino acid was coupled by removal of the Fmoc group 8.8.5 and addition of Fmoc-Pro-OH (0.83 g, 2.4 mmol) as described before 8.8.3. The polymer-bound tetra-peptide was washed exhaustively with

dimethylformamide and dichloromethane and dried *in vacuo.* (91 μmol g^{-1} by Fmoc analysis 8.2.6.1, L_{max}= 0.60 mmol g^{-1}, 15% loading).

An aliquot of resin **256** (3.5 mg) was cleaved under acidic conditions 8.2.7.1. The residue obtained was dissolved in acetonitrile/water (1:1) and analysed by ES-MS as described in section 8.2.8.

m/z (ES-MS; +ve) 659 (100%, MNa^+), 677 (50%, $M+CH_3CN^+$).

8.8.9 *Tetrapeptide bound to 1-[1-({2S}-amino-1-benzyloxycarbonyl-3-phenyl-propyl)-1H-benzotriazole]-carboxyamino-polystyrene 328*



General procedure 8.8.1 was followed using **293** (assume 1.23 mmol g^{-1}, 0.26 g, 0.32 mmol), tetra*kis*(triphenylphosphine)palladium (0) (0.04 g, 0.03 mmol) and borane-dimethyl amine complex (0.11 g, 1.9 mmol) in dichloromethane (10 mL). The deprotection procedure was repeated to give the title compound as brown beads. General procedure 8.8.3 was followed using (*S*)-Fmoc-Pro-OH (0.54 g, 1.6 mmol), free amine resin (0.26 g, 0.32 mmol), 0.45 M HBTU/HOBt (3.5 mL, 1.6 mmol) and diisopropylethylamine (0.45 mL, 2.6 mmol) to obtain the Fmoc-Pro-coupled peptide (0.38 mmol g^{-1}, L_{max} = 0.65 mmol g^{-1}, 58% loading by Fmoc analysis 8.2.6.1) . The Fmoc group was removed using general procedure 8.8.5 and Fmoc-Ala-OH (0.53 g, 1.6 mmol) was coupled by repeating procedure 8.8.3 using similar quantities of reagents. The final amino acid was coupled by removal of the Fmoc group 8.8.5 and addition of Fmoc-Pro-OH (0.54 g, 1.6 mmol) as described before 8.8.3. The

polymer-bound tetra-peptide was washed exhaustively with dimethylformamide and dichloromethane and dried *in vacuo.* (0.38 mmol g^{-1} loading by Fmoc analysis 8.2.6.1, L_{max}= 0.58 mmol g^{-1}, 65% loading).

υ_{max} (KBr disc)/ cm^{-1} 3058, 3023 (CH, aryl), 2922 (sat. CH), 1730 (CO ester and urethane). 1652 (CO, amide I), 1520 (CO, amide II).

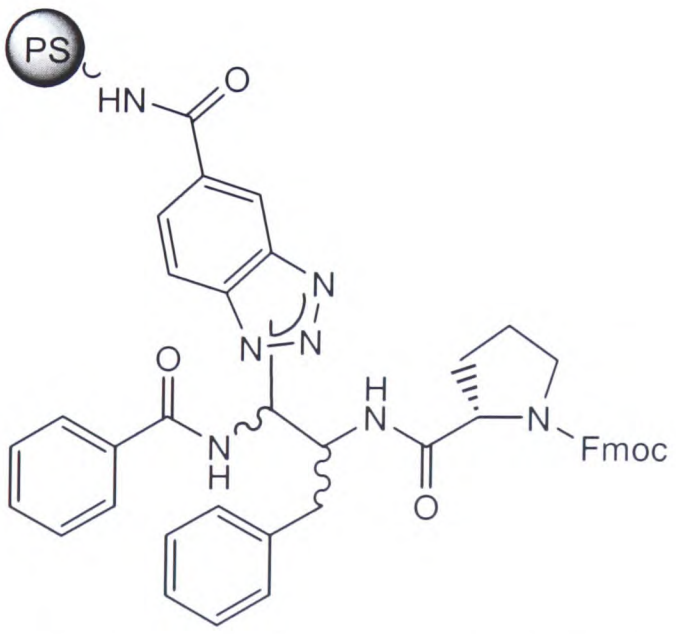
An aliquot of resin **328** (3.5 mg) was cleaved under basic conditions 8.2.7.1 using 1 M NaOH/Dioxane (1:3). The residue obtained was dissolved in acetonitrile/water (1:1) (5 mL) in volumetric flask and analysed by reversed phase HPLC (CH_3CN/H_2O/TFA 30:70:0.01; 21 °C) as described in section 8.2.9.

RP-HPLC; R_t = 7.4 min, benzoic acid; R_t = 9.0 min, peptide aldehyde (product collected and identity confirmed by ES-MS)

m/z (ES-MS; +ve) 637 (60%, MH^+), 659 (100%, MNa^+), 675 (5%, MK^+).

Basic cleavage 8.2.7.2 was repeated however no cleavage products were detected

8.8.10 Dipeptide bound to racemic 1-[1-(amino-1-benzylamino-3-phenyl-propyl)-1H-benzotriazole]-carboxyamino-polystyrene 251

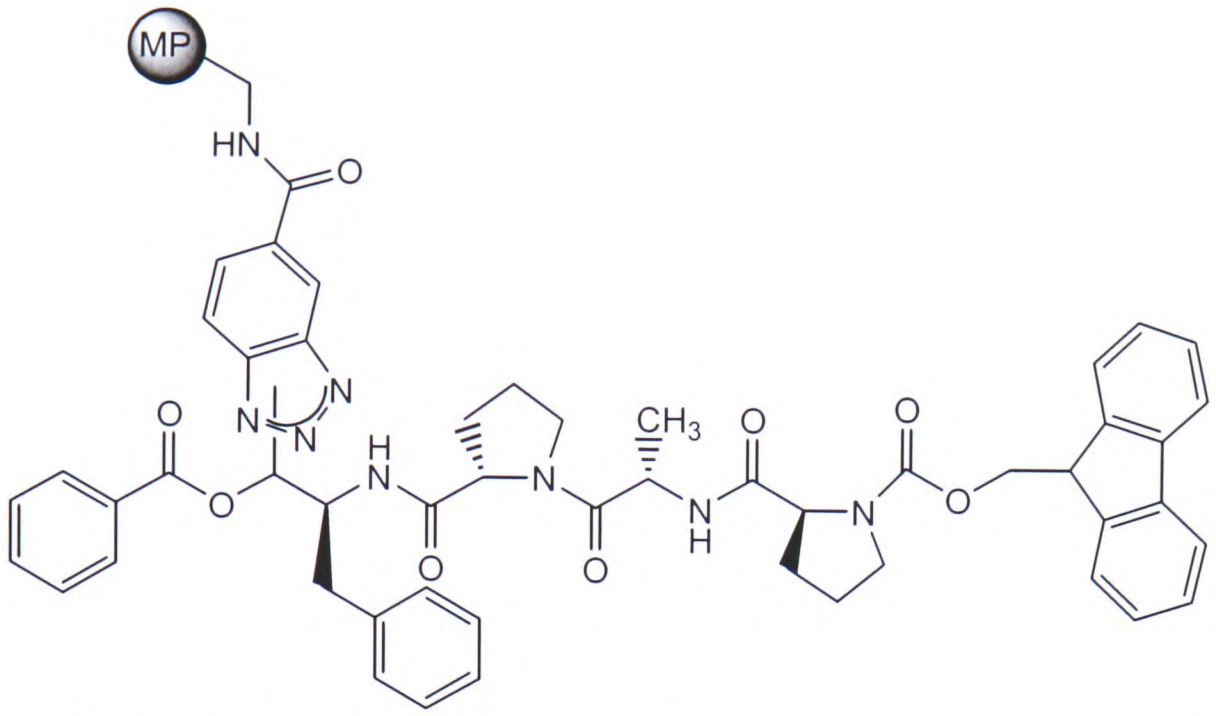


General procedure 8.6.10 was followed using (*R/S*)-**173** (1.8 g, 7.8 mmol, 5eq.), benzamide (0.94 g, 7.8 mmol, 5eq.), *p*-toluene sulfonic acid (70 mg, 0.39 mmol) and **218** (assume 1.23 mmol g^{-1}, 1.27 g, 1.56 mmol, 1eq.) in anhydrous toluene (50 mL). The resin was filtered and washed exhaustively with dichloromethane and dimethylformamide before carrying out the next step. General procedure 8.8.1 was

followed by reacting the diastereomeric resin (assume 1.23 mmol g^{-1}, 0.28 g, 0.34 mmol) with tetra*kis*(triphenylphosphine)palladium (0) (40 mg, 0.034 mmol) and borane-dimethyl amine complex (0.12 g, 2.0 mmol) in dichloromethane (3.5 mL). The deprotection procedure was repeated to give the title compound as brown beads. General procedure 8.8.3 was followed using (*S*)-Fmoc-Pro-OH (0.58 g, 1.7 mmol), free amine resin (0.28 g, 0.34 mmol), 0.45 M HBTU/HOBt (3.8 mL, 1.7 mmol) and diisopropylethylamine (0.47 mL, 2.7 mmol) to obtain the title compound (209 μmol g^{-1}, L_{max}=0.59 mmol g^{-1}, 35% loading)

An aliquot of resin **251** (30 mg) was cleaved under acidic conditions 8.2.7.1. The residue obtained was dissolved in acetonitrile/water (1:1) (2 mL) and analysed by electrospray mass spectrometry as described in Section 8.2.8. *m/z* (ES-MS; +ve) 491 (70%, MNa^+), 572 (100%, $M+104^+$), 612 (45%, $M+144^+$). Purification of the residue was attempted to remove the unexpected mass ions at 572 and 612 *m/z*. Semi-preparative TLC was carried out on an aluminium backed TLC place eluting with 1:1 ethylactate/hexane, which revealed the products; R_f 0.71, 0.33 and baseline product. The desired product at R_f = 0.33 was collected and freeze dried before being analysed by electrospray mass spectrometry and high resolution ^1H NMR. *m/z* (ES-MS; +ve) 491 (70%, MNa^+), 572 (100%, $M+104^+$), 612 (45%, $M+144^+$), 693 (15%, $M+225^+$), 715 (20%, $M+247^+$); δ_H ($CDCl_3/CD_3OD$; 600 MHz) 9.63 (1H, C*H*O), 9.95 (1H, C*H*O).

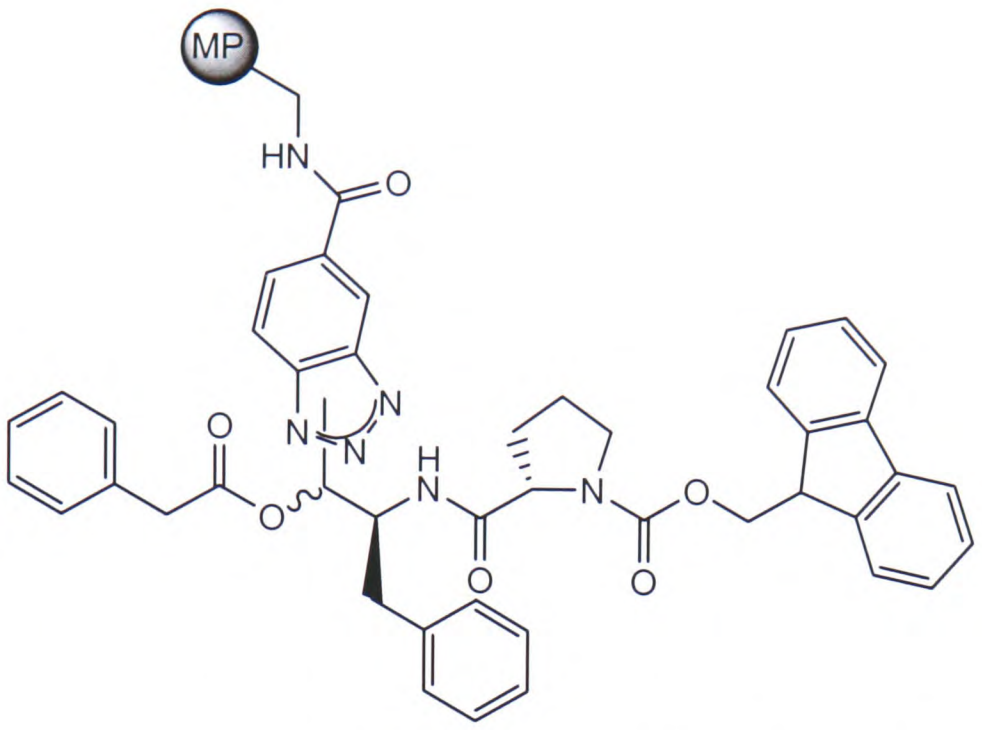
8.8.11 *Tetrapeptide bound to 1-[1-({2S}-amino-1-benzyloxycarbonyl-3-phenyl-propyl)-1H-benzotriazole]-carboxyamino-macroporous polystyrene 329*



General procedure 8.8.1 was followed using 1-[1-({2S}-allyloxycarbonylamino-1-benzyloxycarbonyl-3-phenyl-propyl)-1*H*-benzotriazole]-carboxyamino-macroporous polystyrene (assume 1.19 mmol g^{-1}, 480 mg, 580 μmol), tetra*kis*(triphenylphosphine)palladium (0) (0.07 g, 0.06 mmol) and borane-dimethyl amine complex (0.20 g, 3.5 mmol) in dichloromethane (10 mL). The deprotection procedure was repeated to give the title compound as pale brown beads. General procedure 8.8.3 was followed using (*S*)-Fmoc-Pro-OH (0.99 g, 2.9 mmol), free amine resin (0.48 g, 0.58 mmol), 0.45 M HBTU/HOBt (6.5 mL, 2.9 mmol) and diisopropylethylamine (0.82 mL, 4.7 mmol) to obtain the Fmoc-Pro-coupled peptide. The Fmoc group was removed using general procedure 8.8.5 and Fmoc-Ala-OH (0.97 g, 2.95 mmol) was coupled by repeating procedure 8.8.3 using similar quantities of reagents. The final amino acid was coupled by removal of the Fmoc group 8.8.5 and addition of Fmoc-Pro-OH (0.99 g, 2.9 mmol) as described in section 8.8.3. The polymer-bound tetra-peptide was washed exhaustively with dimethylformamide and dichloromethane and dried *in vacuo.* (0.20 mmol g^{-1} loading by Fmoc analysis 8.2.6.1, L_{max}= 0.58 mmol g^{-1}, 35% loading).

υ_{max} (KBr disc)/ cm^{-1} 3020 (CH, aryl), 2925 (sat. CH), 1718 (CO, ester and urethane). 1643 (CO, amide I), 1604 (CH, aryl), 1510 (CO, amide II).

8.8.12 *Dipeptide bound to 1-[1-({2S}-amino-1-phenylacetyloxycarbonyl-3-phenyl-propyl)-1H-benzotriazole]-carboxyamino-macroporous polystyrene 330*



General procedure 8.8.1 was followed using 1-[1-({2S}-allyloxycarbonylamino-1-phenylacetyloxycarbonyl-3-phenyl-propyl)-1*H*-benzotriazole]-carboxyamino-macroporous polystyrene (assume 1.19 mmol g^{-1}, 0.43 g, 0.51 mmol), tetra*kis*(triphenylphosphine)palladium (0) (0.06 g, 0.05 mmol) and borane-dimethyl amine complex (0.18 g, 3.1 mmol) in dichloromethane (7 mL). The deprotection procedure was repeated to give the title compound as pale brown beads. General procedure 8.8.3 was followed using (*S*)-Fmoc-Pro-OH (0.80 g, 2.4 mmol), free amine resin (0.40 g, 0.47 mmol), 0.45 M HBTU/HOBt (5.2 mL, 2.4 mmol) and diisopropyl ethylamine (0.66 mL, 3.8 mmol) to obtain the Fmoc-Pro-coupled peptide. The polymer-bound dipeptide was washed exhaustively with dimethylformamide and dichloromethane and dried *in vacuo.* (0.14 mmol g^{-1} loading by Fmoc analysis 8.2.6.1, L_{max}= 0.63 mmol g^{-1}, 22% loading).

υ_{max} (KBr disc)/ cm^{-1} 3020 (CH, aryl), 1720 (CO urethane and ester). 1642 (CO, amide I), 1510 (CO, amide II).

Appendix A: Summary of Biocatalysis in Polymer-Supported synthesis/hydrolysis

Solid support	Enzyme	Time	MWt	Comments
PEGA?	Subtilisin Carlsberg	24 h	27 kDa	Quantitative cleavage[26]
Kieselguhr/ polydimethylacrylamide	Subtilisin Carlsberg	24 h	27 kDa	Quantitative cleavage[26]
PEGA	leukocyte antigen receptor phosphatase	60 min	35 kDa	Near quantitative dephosphorylation[28]
PEGA	Chymotrypsin	45 min	22 kDa	Cleavage observed[28]
Kieselguhr	leukocyte antigen receptor PTP	60 min	35 kDa	Near quantitative dephosphorylation[28]
Kieselguhr	Chymotrypsin	45 min	22 kDa	Cleavage observed[28]
TentaGel	leukocyte antigen receptor PTP	60 min	35 kDa	2% dephosphorylation[28]
PEGA	Trypsin	26 h	23 kDa	Most of the material bound to the resin was accessible to the enzyme[29]
TentaGel	Trypsin	26 h	23 kDa	No enzymatic cyclisation or hydrolysis detected[29]
$PEGA_{1900}$	*E. coli* leader peptidase	18 h	37 kDa	FRET based assay, cleavage observed[30]
$PEGA_{1900}$	Napsin A	18 h	38 kDa	FRET based assay, cleavage observed[30]
$PEGA_{6000}$	MMP-9	40 h	67/83 kDa	FRET based screening of peptide libraries. No cleavage yields given[31]
$PEGA_{4000}$	MMP-9	7 h	67/83 kDa	FRET based screening of peptide libraries. <10% cleavage[31]
$PEGA_{1900}$	MMP-12	22 h	22 kDa	FRET assay – cleavage observed[32]
ArgoGel	Papain	Prolonged treatment	23 kDa	Low hydrolysis yields[35]
TentaGel	Papain	Prolonged Treatment	23 kDa	Low hydrolysis yields[35]
PEGA?	Papain	1 h	23 kDa	Near quantitative

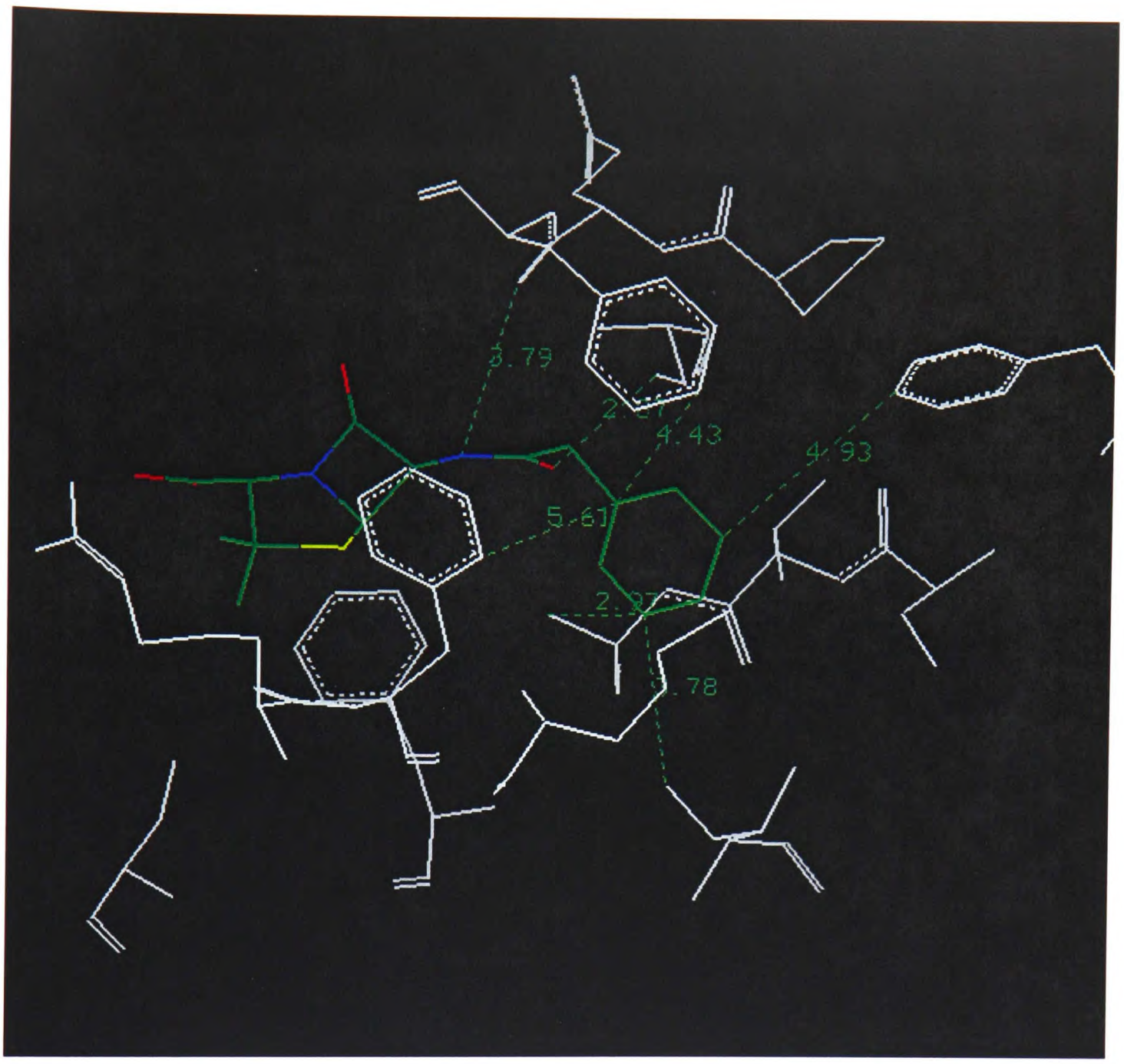
				cleavage of peptide substrate[35]
$PEGA_?$	α-chymotrypsin	5 h	22 kDa	Cleavage observed[35]
$PEGA_{4000}$ (300-400μm)	Papain	>3.5 h	23 kDa	FRET assay - maximum 52% cleavage of peptides[36]
$PEGA_?$	Trypanothione reductase	O\N	55 kDa	Cleavage observed[25]
TentaGel	Trypanothione reductase	O\N	55 kDa	no cleavage observed[25]
$EXPO_{3000}$	Subtilisin Carlsberg	-	27 kDa	No cleavage yields given[37]
$PEGA_{1900}$	β–1,4–galactosyltransferase	48 h	50 kDa	95% enzymatic synthesis[17]
$PEGA_{1900/300}$	β–1,4–galactosyltransferase	72 h	50 kDa	50% enzymatic synthesis[17]
Polyacrylamide (soluble)	α–2,3-sialyltransferase	3 d	52 kDa	>99% enzymatic synthesis[22]
Polyacrylamide (soluble)	Ceramide glycanase	17 h	-	61% enzymatic synthesis[22]
CPG	β–1,4–galactosyltransferase	48 h	50 kDa	>98% enzymatic synthesis [23]
CPG	α–2,3-sialyltransferase	48 h	52 kDa	>98% enzymatic synthesis [23]
TentaGel	Elastase	long cleavage time	26 kDa	10-15% cleavage[24]
TentaGel	Pepsin A	long cleavage time	35 kDa	10-15% cleavage[24]
TentaGel	chymotrypsin	Long cleavage time	22 kDa	2-2.5% cleavage[24]
TentaGel	Streptavidin	-	60 kDa	surface cleavage only[24]
TentaGel	Anti-β-endorphin antibody	-	17 0kDa	surface cleavage only[24]
TentaGel	Thrombin	-	35 kDa	surface cleavage only[24]
$PEGA_{800}$	Cathepsin B	20 h	52 kDa	Cleavage observed[27]
$PEGA_{800}$	Cathepsin C	14 h	56 kDa	Cleavage observed[27]
$PEGA_{1900}$	Cruzipain	24 h	57 kDa	Only partial cleavage observed,

				mainly at surface[34]
$PEGA_{4000}$	recombinant cysteine protease from *Leishmania mexicana* CPB2.8ΔCTE	7 h	-	Cleavage observed[33]
CPG	Penicillin acylase	2 h	80 kDa	Quantitative cleavage[40]
Polymer membrane	Trypsin	3 h	23 kDa	Cleavage observed[38]
kieselguhr/ polydimethylacrylamide	T4 RNA ligase	slow	44 kDa	Synthesis observed[42]
kieselguhr/ polydimethylacrylamide	RNase A	-	14 kDa	Cleavage observed[42]
Aminopropyl silica	α-chymotrypsin	-	22 kDa	95% cleavage[41]
Aminopropyl silica	α-2,3-sialyltransferase	-	52 kDa	65% synthesis [41]
Aminopropyl silica	β-1,4-galactosyltransferase	-	50 kDa	55% synthesis[41]
TentaGel	Penicillin acylase	48 h	80 kDa	1% [43,44]
$PEGA_{1500}$	Penicillin acylase	48 h	80 kDa	13% [43,44]
CPG (pore size 50nm)	Penicillin acylase	48 h	80 kDa	10% [43,44]
POE_{6000}	Penicillin acylase	48 h	80 kDa	59% [43,44]
Polyacrylamide(soluble)	a-chymotrypsin	24 h	22 kDa	72% overall synthesis[45,46]
Polyacrylamide(soluble)	α-2,3-sialyltransferase	48 h	52 kDa	Synthesis observed[45,46]
Polyacrylamide(soluble)	β-1,4-galactosyltransferase	24 h	50 kDa	Synthesis observed[45,46]
SPOCC	Subtilisin BNP'	30 min	27 kDa	FRET based screening – cleavage observed[47]
SPOCC	MMP-9	24 h	72 kDa	No cleavage observed[47]
Pepsin K (polyacrylamide)	Calf spleen phosphodiesterase	7 d	-	84% cleavage[48]
TentaGel	Recombinant lipase RB001-05	-	-	70-80% cleavage[49]
TentaGel	Penicillin acylase	16 h	80 kDa	50% cleavage[50]
Sepharose 6B	β-1,4-galactosyltransferase	5 d	50 kDa	98% enzymatic synthesis[21]
Sepharose 6B	α-2,3-sialyltransferase	5 d	52 kDa	75% enzymatic synthesis[21]
Sepharose 6B	α-1,3/4-fucosyltransferase	5 d	42 kDa	95% enzymatic synthesis[21]
TentaGel	MMP-12	O\N	22 kDa	No cleavage[14]
TentaGel	Thermolysin	O\N	35 kDa	No cleavage[14]
TentaGel	MMP-13	O\N	42.5 kDa	No cleavage[14]
TentaGel	Clostridium collagenase	O\N	68 kDa	No cleavage[14]
TentaGel	NEP	O\N	90 kDa	No cleavage[14]

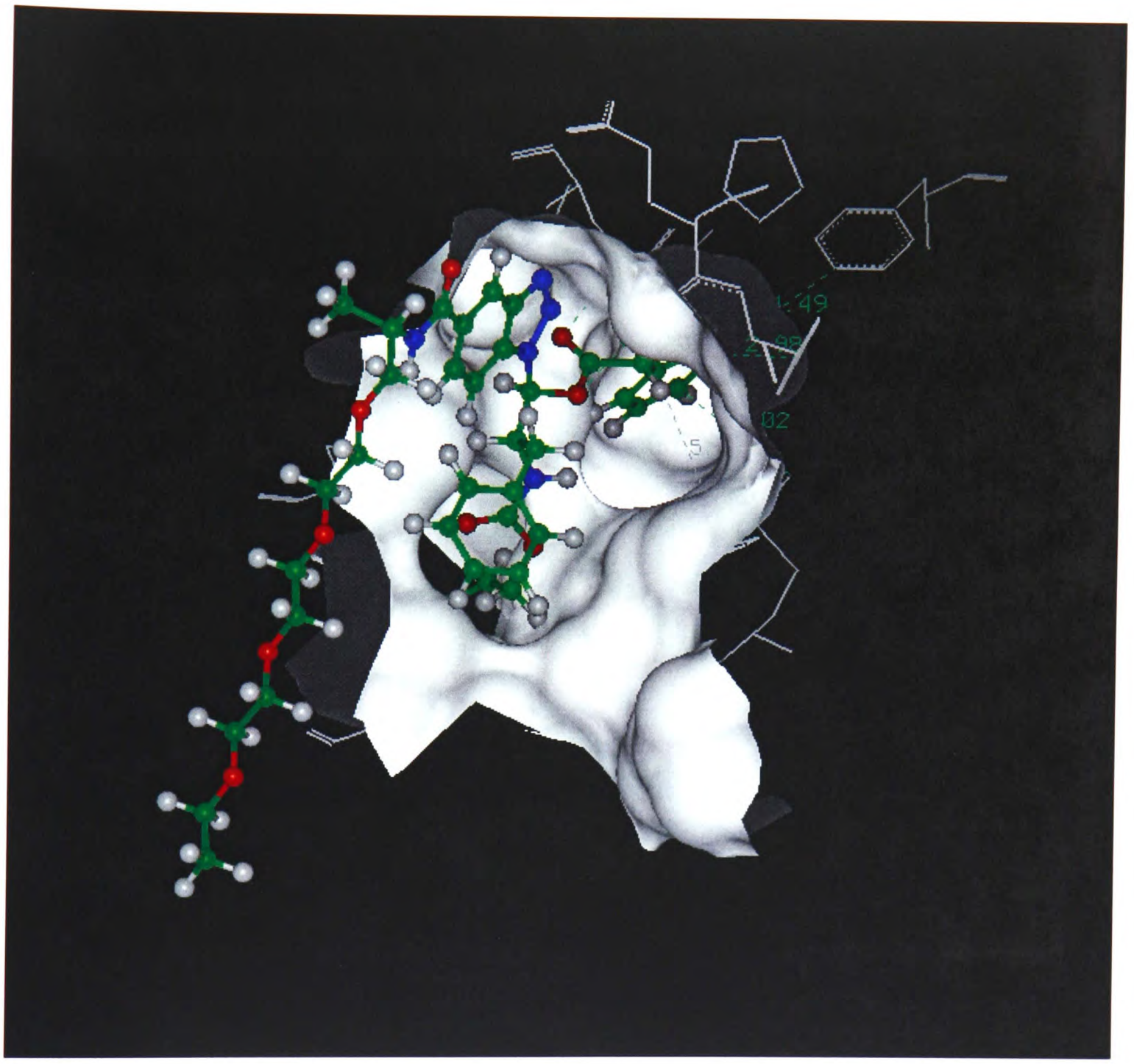
$PEGA_{1900}$	MMP-12	O\N	22 kDa	Cleavage observed[14]
$PEGA_{1900}$	Thermolysin	O\N	35 kDa	Cleavage observed[14]
$PEGA_{1900}$	MMP-13	O\N	42.5 kDa	No cleavage[14]
$PEGA_{1900}$	Clostridium collagenase	O\N	68 kDa	No cleavage[14]
$PEGA_{1900}$	NEP	O\N	90 kDa	No cleavage[14]
CPG (pore size 100nm)	MMP-12	O\N	22 kDa	Cleavage observed[14]
CPG (pore size 100nm)	Thermolysin	O\N	35 kDa	Cleavage observed[14]
CPG (pore size 100nm)	MMP-13	O\N	42.5 kDa	Cleavage observed[14]
CPG (pore size 100nm)	Clostridium collagenase	O\N	68 kDa	Cleavage observed[14]
CPG (pore size 100nm)	NEP	O\N	90 kDa	Cleavage observed[14]

Appendix B: Space filled molecular model illustrating Penicillin G within the active site of penicillin acylase.

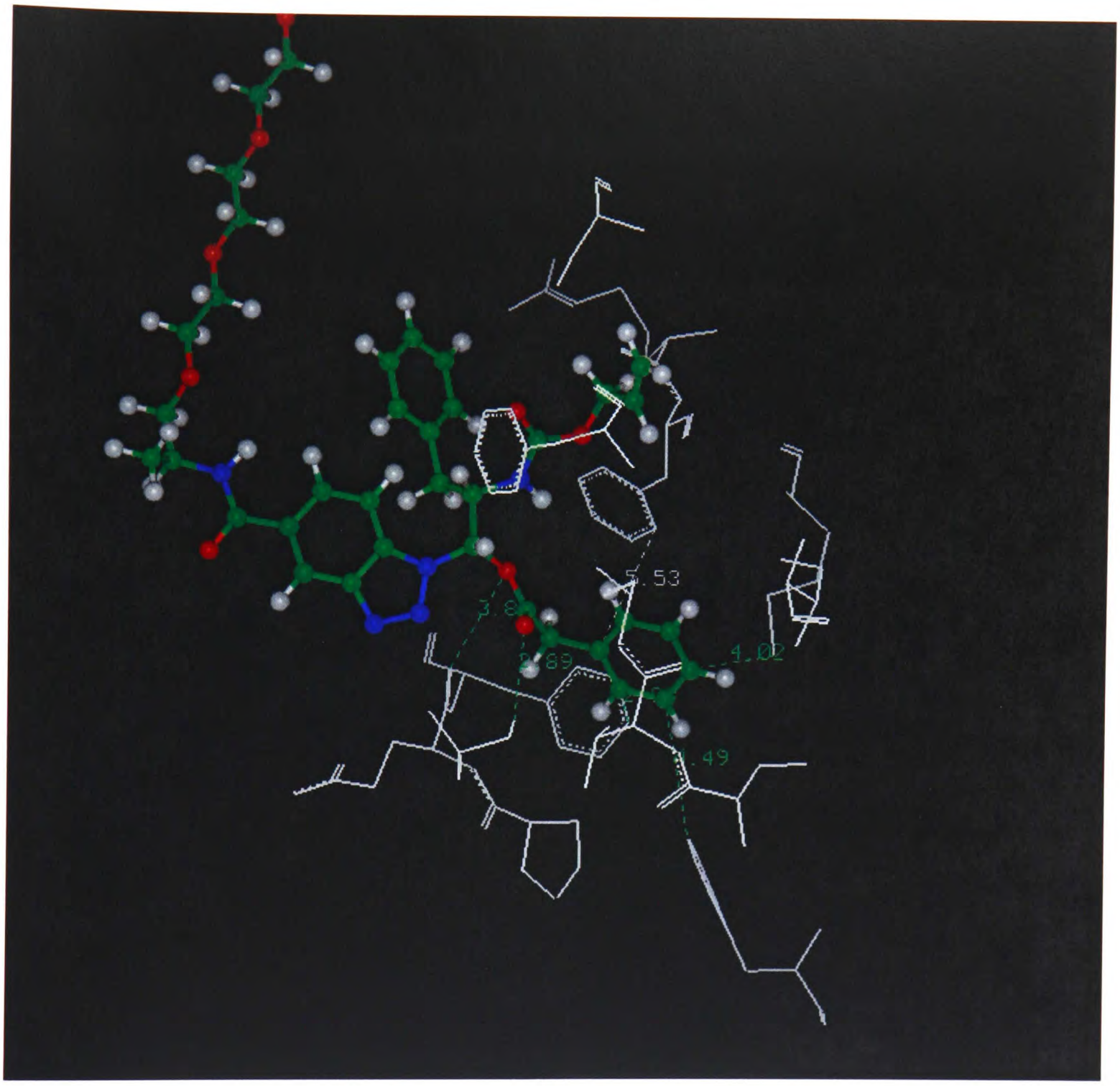
Appendix C: Molecular model of Penicillin G in active site showing possible important interactions.



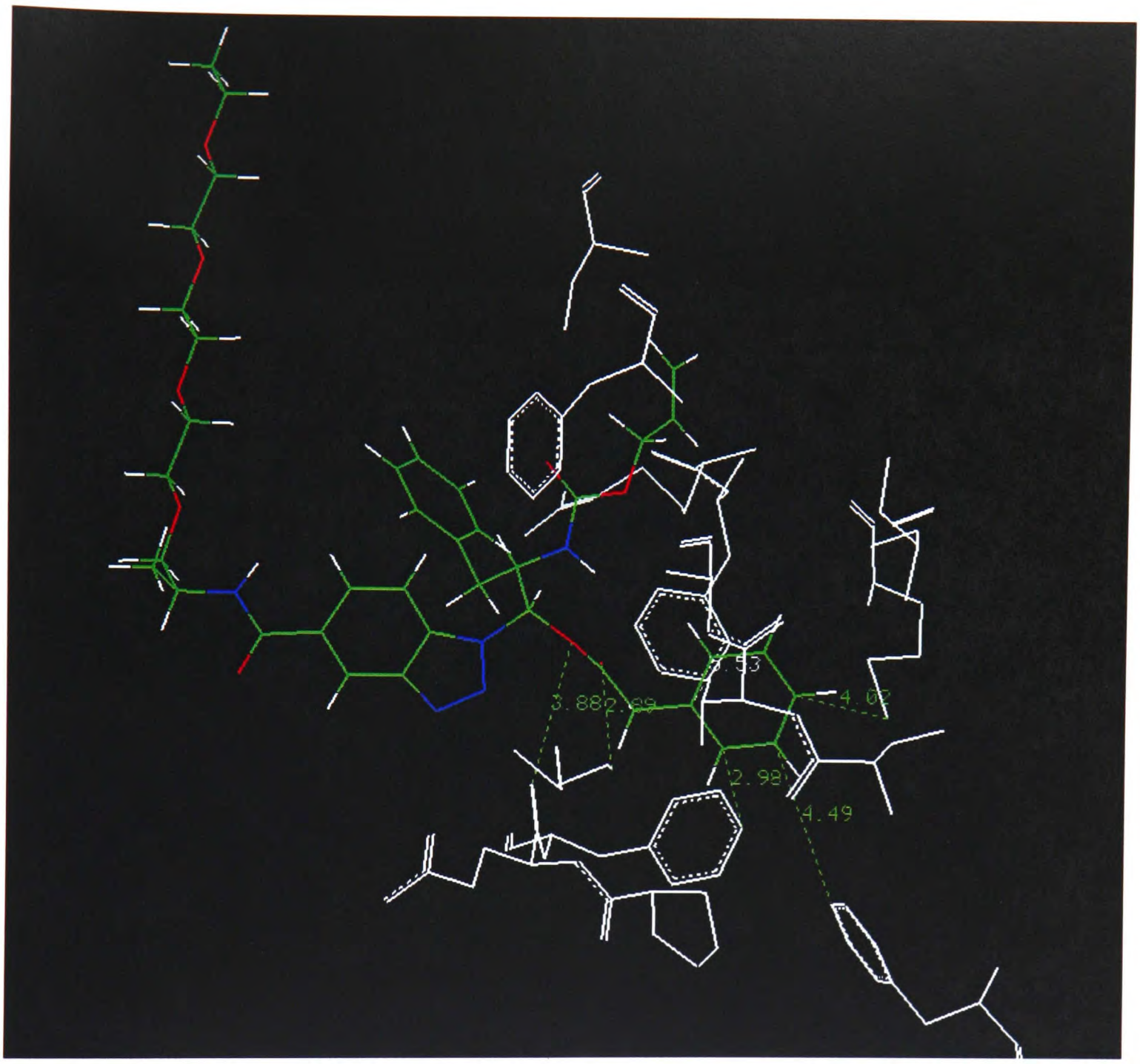
Appendix D: Molecular model depicting possible favourable interactions between (R,S)-linker 296 and hydrophobic binding pocket of penicillin acylase.



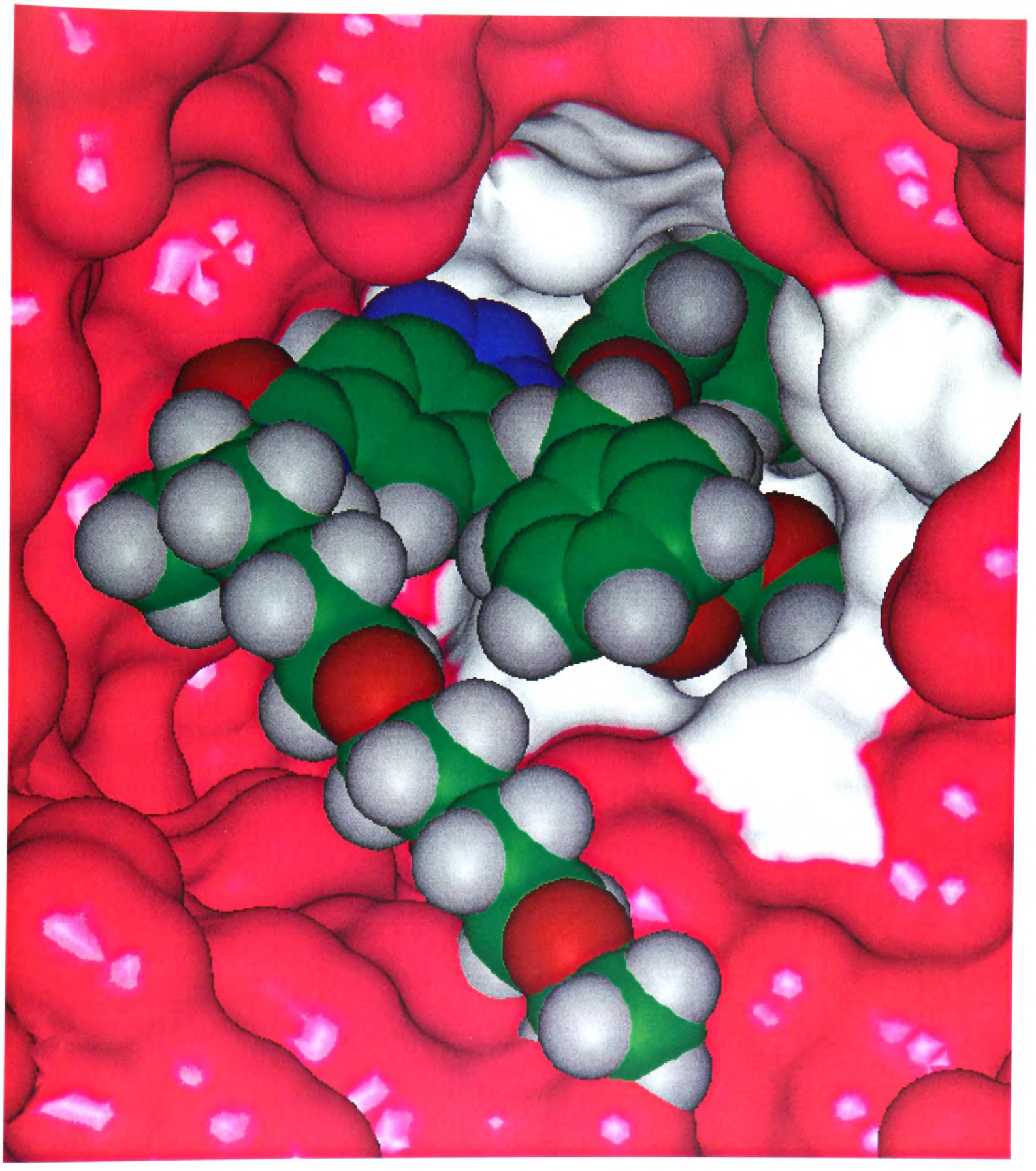
Appendix E: Molecular model of (R,S)-linker 296 showing possible important interactions with the active site.



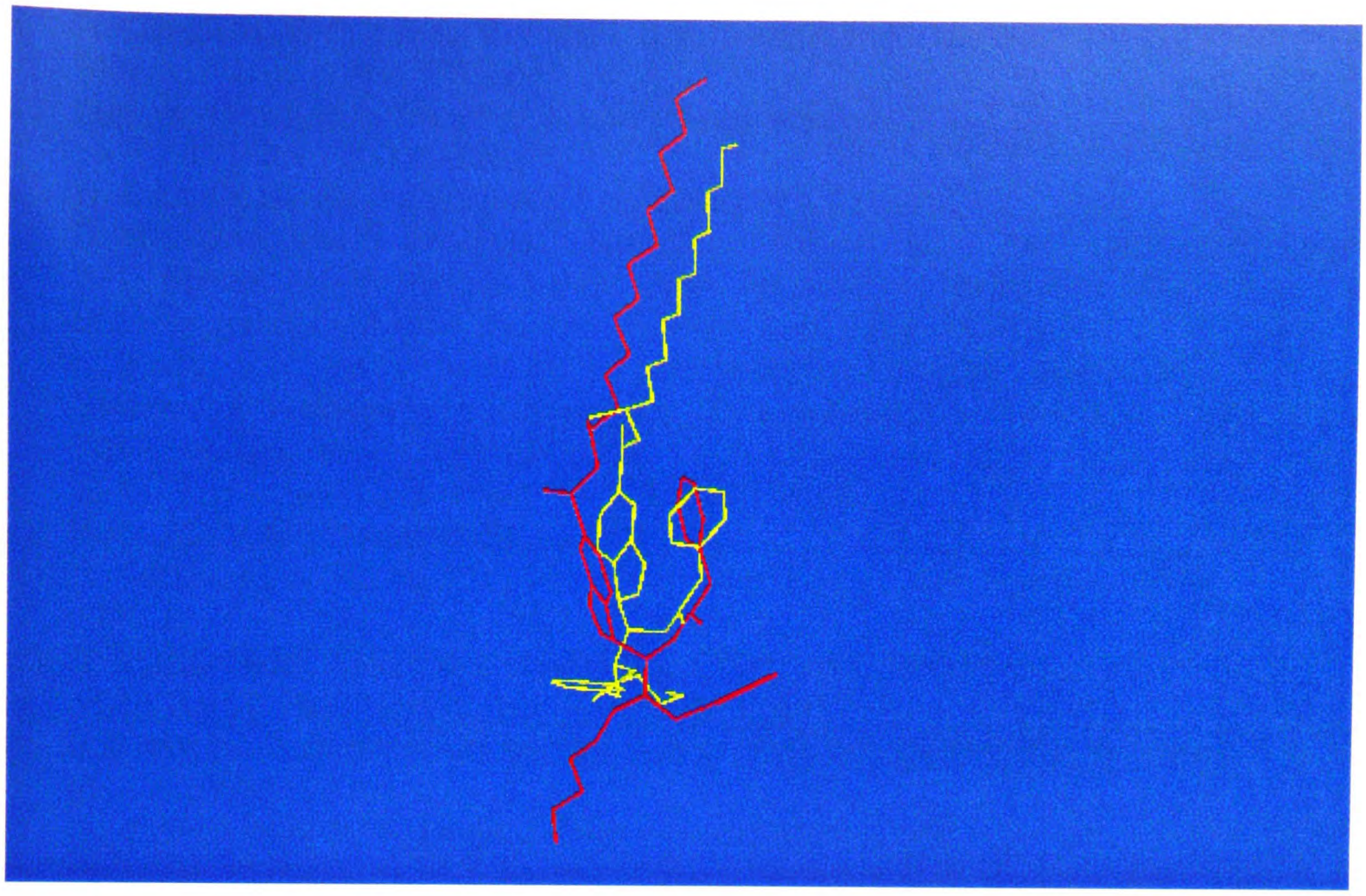
Appendix F: Molecular model of (R,S)-linker 296 in active site of penicillin acylase.



Appendix G: Space-filled molecular model of (R,S)-linker 296 in active site of penicillin acylase.



Appendix H: Chem3D model of the two diastereomers of linker 296. [(S, S)-diastereomer = red; (R,S)-diastereomer = yellow]



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